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TESIS DOCTORAL

Physiological strategies in orophytes from Sierra de Guadarrama in response to harsh environments

Estrategias fisiológicas de respuesta a ambientes adversos en orófitos de la Sierra de Guadarrama

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

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Madrid

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Madrid, 2019

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La flor que crece en la adversidad
es la más rara y hermosa de todas

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RESUMEN

ANTECEDENTES

La predicción de cambio en los parámetros climáticos (disminución de las precipitaciones, aumento de las temperaturas), aunado al aumento de la perturbación antrópica, resaltan la fragilidad y dificultad de supervivencia de la gran diversidad de especies vegetales colonizadoras de los ecosistemas de la alta montaña Mediterránea. La exigüidad de los estudios enfocados en las respuestas fisiológicas y la capacidad adaptativa de las especies que forman la vegetación de las cumbres Mediterráneas es muy alta, comparado con los crecientes estudios de esta índole en sistemas alpinos. Esto destaca la necesidad de identificar las respuestas específicas para contrarrestar los efectos de la sequía estival, incidente a lo largo de la corta temporada de actividad vegetal, en las especies que integran estas comunidades de alta montaña. Estos estudios ayudarán a discernir posibles alteraciones en la estructura de estas comunidades y, dado el esperado aumento en la intensidad de la sequía estival en estos ecosistemas, facilitar su conservación.

OBJETIVOS

El objetivo principal de esta Memoria doctoral es el estudio de los mecanismos fisiológicos de respuesta al estrés estival en orófitos de las comunidades de la alta montaña Mediterránea; incluyendo especies endémicas y de limitada distribución, como es el caso de *Armeria caespitosa,* o *Erysimum penyalarense.* Para ello, se llevó a cabo la cuantificación estacional de metabolitos ampliamente estudiados en especies modelo y cultivadas, establecidos como indicadores de la respuesta al estrés por sequía y temperaturas extremas (osmolitos: prolina y carbohidratos no estructurales, NSC en inglés). Aunado a esto, se estudió el papel de pigmentos fotosintéticos y fotoprotectores en estos orófitos, esclareciendo sus oscilaciones en respuesta a la variación climática estacional y diaria, respectivamente. Finalmente, buscando evaluar la capacidad adaptativa de la estructura foliar en respuesta al agravamiento de las condiciones climáticas registrado en estas montañas, se evaluaron caracteres relacionados con la actividad fotosintética y termorregulación en pliegos de herbario de los últimos 71 años.

RESULTADOS Y CONCLUSIONES

La gran diversidad en las estrategias de respuesta al estrés encontrada tanto entre especies como entre los mismos grupos funcionales confirma la gran capacidad adaptativa de estos orófitos, atribuida a la presencia y severidad de los gradientes medioambientales propios de estos hábitats de alta montaña. A su vez, cabe resaltar que la respuesta específica de cada especie fue relacionada con sus estrategias de desarrollo, modulada por la intensidad del estrés estival.

La extensiva revisión bibliográfica realizada permitió contabilizar la diversidad de respuestas fisiológicas factiblemente instauradas en los orófitos seleccionados; así como las posibles

adaptaciones en los mismos en respuesta al cambio en los parámetros climáticos sobrevenido en estos hábitats. A su vez, fue posible vislumbrar la asidua brecha de conocimiento entre la ampliamente estudiada fisiología vegetal de especies alpinas, contrastando con la de especies de bioclima Mediterráneo. Esta revisión permitió la puesta a punto de los estudios que conforman de la presente Memoria doctoral.

La acumulación significativa de los osmolitos prolina y NSC, en los orófitos estudiados demostró jugar un papel crucial en su respuesta ante el estrés por sequía estival, acrecentado durante el transcurso de la temporada de crecimiento. A su vez, se identificó una predilección por la acumulación de prolina en respuesta a dicho estrés, indicándolo como el principal osmolito estudiado involucrado en la respuesta a la sequía estival en estos orófitos. Conjuntamente, el aumento de inulina y fructosa durante una temporada de crecimiento atípicamente cálida y seca (i.e. 2017), permite sugerir a la prolina y NSC como estrategias complementarias de respuesta a la sequía estival severa en estos orófitos.

El aumento en la intensidad del estrés estival a lo largo de la temporada de crecimiento instigó la activación de mecanismos de fotoprotección en herbáceas de la alta montaña Mediterránea, i.e. activación del ciclo de las xantofilas y, factiblemente, del ciclo de la luteína. La significativa acumulación de compuestos de-epoxidados, principalmente zeaxantina, en individuos de altitudes contrastantes, permite calificar de imprescindible la activación de mecanismos fotoprotectores en las herbáceas de alta montaña mediterránea. Igualmente, el constantemente elevado contenido de luteína encontrado en estas especies sugiere la intervención del ciclo de la luteína como una fuente de fotoprotección continua independiente de la variación diurna en factores climáticos, a diferencia del ciclo de las xantofilas. Aunado a la presencia de superiores estrategias fotoprotectoras en estos orófitos, los ajustes en la morfología foliar y estomática en respuesta a la transición hacia condiciones áridas registrada en la Sierra de Guadarrama; infieren una gran capacidad adaptativa en *Senecio carpetanus* al agravamiento climático previsto para estas zonas. Lo anterior gracias a la selección de atributos que favorecen su óptimo funcionamiento en ambientes adversos (i.e. reducción del área foliar y estomática, mayor densidad de estomas), relacionado a su naturaleza como especie subordinada en estas comunidades de alta montaña.

En general, podemos concluir que existe una resiliencia de las comunidades de la alta montaña a los efectos del agravamiento en las condiciones meteorológicas, consecuencia del cambio climático; sin dejar de suponer una posible alteración en su estructura y dinámicas a causa de la observada variación en la sensibilidad particular de cada especie. Por ejemplo, con relación a mecanismos fotoprotectores, la significativa reducción de clorofila en estos orófitos en respuesta a las olas de calor de 2017, sumada a la falta de un aumento significativo en pigmentos fotoprotectores para contrarrestar una inactivación del altamente termolábil sistema fotosintético

bajo dichos episodios de estrés intenso. A su vez, la significativa acumulación de prolina ante recurrentes episodios climáticos extremos por parte de especies dominantes y tolerantes a sequía (*Festuca curvifolia* y *Juniperus communis* subsp. *alpina*), resalta la existencia de una inminente fragilidad delimitada por la severidad, frecuencia y duración de dichos eventos extremos durante la temporada de crecimiento. En consecuencia, es necesario continuar los esfuerzos para refinar el límite y alcance de los mecanismos que confieren dicha protección a estos orófitos bajo condiciones adversas (olas de calor, retiro prematuro de cobertura de nieve), en vista del pronosticado aumento de las temperaturas; aunado al agravamiento y extensión de la temporada de sequía en la cuenca Mediterránea, cuyo grave impacto en la fenología y fisiología de la vegetación ha sido resaltado en estudios previos.

SUMMARY

BACKGROUND

The impending changes in climatic conditions (i.e. decrease in rainfall regimes and increasing temperatures) as an outcome of climate change, coupled to a greater incidence of human disturbance, underlines the challenges and vulnerability faced by Mediterranean high-mountain plant communities. Albeit increased research efforts have demonstrated the effects of environmental factors over plant physiology, along with the responses and adaptation capacity on high-mountain plant species; these primarily concern the vegetation from temperate mountains (i.e. alpine), while studies on Mediterranean high-mountain flora, including their Spanish elements, remain scarce. The latter exposes the urgent need to identify the specific responses and strategies to counteract the seasonal aggravation of summer stress in the remaining species comprising these Mediterranean high-mountain communities. This in view of the expected lengthening and increased intensity of the summer season in these mountains. In turn, these studies will enable discerning potential alterations in community structure and dynamics, fostering their conservation.

OBJECTIVES

The main objective of the present PhD Thesis is to study the physiological mechanisms driving the response to adverse conditions in high-mountain specialists and narrowly-distributed species inhabiting the Sistema Central mountainous range, for instance *Armeria caespitosa* and *Erysimum penyalarense*. Specifically, we aimed to unveil the strategies in response to the main abiotic stressor driving plant life in Mediterranean high-mountains, i.e. summer stress. In turn, we assessed the involvement of well-known responses in lowland species. For instance, osmolyte accumulation (proline and non-structural carbohydrates, NSC) in response to drought and occasional freezing stress befalling the brief growing season. In addition, we evaluated the protective role of photosynthetic and "accessory" pigments in response to the seasonal aggravation of summer stress and diurnal climatic variations, respectively. Lastly, we assessed the adaptive capacity of a high-mountain hemicryptophyte using herbarium specimens from the past 71 years to the long-term climate exacerbation in these high-mountain areas, via morphological and micro-morphological leaf traits related to transpiration-mediated cooling and photosynthetic gain.

RESULTS AND CONCLUSIONS

The diversity in stress-response strategies found among the studied high-mountain specialists ratifies their superior adaptive capacity in response to the challenging environmental gradients

encountered in high-mountain habitats. These species-specific responses were associated to their life strategies and to the severity of environmental constraints, primarily, summer stress.

The extensive literature review provided an overview of the wide variety in physiological responses and adaptations underlying in the selected high-mountain specialists, granting the background and framework for our upcoming studies. In addition, the substantial knowledge gap found between alpine and Mediterranean high-mountain vegetation revealed the impending need to focus more research endeavors into studying the adaptive responses in Mediterranean high-mountain plants via stress indicators. In view of the foretold aggravation of climatic conditions in Mediterranean high-mountain habitats, this review furthered the objectives and relevance of this PhD research.

The role of osmolyte accumulation appeared as a crucial response mechanism to counteract the effects of the seasonal aggravation of summer drought in the studied high-mountain specialists. Our findings revealed a proclivity for proline accumulation in response to summer stress in these species, suggesting proline as the main osmolyte involved in the drought-stress response in the studied chamaephytes and hemicryptophytes. However, buildup of other osmolytes such as non-structural carbohydrates (NSC) also contributed to ameliorating the effect of summer drought in hemicryptophytes, particularly during an atypically dry and warm growing season. Hence, these findings suggest NSC and proline act as complementary mechanisms to provide optimal drought endurance in Mediterranean high-mountain orophytes under severe summer stress.

The seasonal exacerbation of summer stress elicited the activation of a series of photoprotective strategies in Mediterranean high-mountain herbs, i.e. the activation of the xanthophyll and lutein cycles. The significantly higher de-epoxidated compounds (mainly zeaxanthin) found in samples from high and low elevations; indicate xanthophyll cycle pigments play a key role in avoiding photoinhibition in these high-mountain herbs. Furthermore, the high lutein levels found in all species, suggests the involvement of the lutein cycle as a sustained photoprotective strategy that, contrary to the xanthophyll cycle, appears as impartial to diurnal fluctuations in climatic factors. The activation of these photoprotective strategies could explain the minor variances in chlorophyll *a/b* ratio to altitude and the seasonal exacerbation of summer stress; suggesting a greater resilience of the photosynthetic apparatus via superior photoprotective strategies, which is crucial to guarantee the optimum response of the photosystem and resource acquisition during the brief growing season in Mediterranean high-mountains. Related to the higher photoprotection in these herbs are the adjustments in leaf and stomatal morphology found in *Senecio carpetanus,* interpreted as a response to the climate exacerbation in Sierra de Guadarrama, intensifying in the last 30 years. This suggests the selection of traits enhancing plant fitness in this species, in

response to the shift towards drier and coarser conditions during the growing season registered in these mountains.

Overall, these findings allow inferring the resilience of these Mediterranean high-mountain plant communities to the current exacerbation of environmental conditions, outcome of climate change. However, the potential alteration of their dynamics and structure based on the high variations in the degree of sensitivity among species, dependent on the intensity of the stress, should not be disregarded. For instance, the significant reduction in chlorophyll exerted in herbaceous species in response to the heatwave events in 2017 and the absence of an increased xanthophyll or lutein content to counteract plausible photosynthetic impairment. In addition, the observed significant responsiveness to extreme climatic episodes in the dominant and drought-tolerant species (e.g. *Festuca curvifolia, Juniperus communis* subsp. *alpina*) via increased osmolyte accumulation highlights the potential liability of tolerant species comprising these plant communities to an increased duration, frequency and severity of extreme climatic events befalling the growing season. In turn, these findings emphasize the need to invest further research endeavors to refine the limit and extent to which these strategies allow counteracting summer stress aggravation in these orophytes. The aforementioned in view of the impending increasing temperatures and, by extension, the coarsening of summer stress as consequence of climate change; whose negative impact over the phenology and physiology in plants from these mountains has been emphasized in previous studies.

INTRODUCTION

High-mountain areas, i.e. regions above the tree line, are regarded as hot spots of biodiversity, hosting nearly 10,000 plant species within approximately 5% of the surface of the earth (Körner, 2003). The interaction of challenging climatic conditions, characteristic of high-mountain environments, gives rise to abrupt environmental gradients within short distances acting as a filter for plant establishment. This assortment of ecological gradients, in combination with a remarkable species diversity and relatively low anthropogenic disturbance, provides outstanding prospects to study the effects of abiotic stresses on plant physiology and related plant responses under natural environments (Körner, 2003).

The effects of abiotic stressors on plant physiology and plant adaptation in high-mountain vegetation has been exposed in multiple studies (see Magaña Ugarte et al. 2019). However, most of these refer to species from mountains with a temperate bioclimate (e.g. alpine areas), while studies on the physiology of Mediterranean high-mountain flora, in particular the one from Sierra de Guadarrama, remain scarce (but see García-Fernández et al. 2013; Pescador et al., 2016). In Mediterranean high-mountains, the concomitant effect of elevated temperatures, high irradiance and water scarcity during summer (i.e. summer stress) which befalls the effective growing season of vegetation, has been deemed as the main constraint for plant performance and survival (García-Camacho and Escudero, 2009; Gutiérrez-Girón and Gavilán, 2013). These conditions contrast to the experienced by their alpine counterparts throughout the growing season, where water availability is not a limiting factor (Körner, 2003). Furthermore, the incidence of both drought and temperature severity gradients in Mediterranean mountains acting in opposite directions (i.e. lower temperatures as altitude increases, drought stress declining with altitude) gives rise to further coercing selective pressures along the elevation gradient, allowing only well-adapted species to thrive (Pescador et al., 2015). This unique set of features present in Mediterranean high-mountain environments grants natural laboratories to explore the effects of climatic exacerbation on the physiology and adaptive responses of high-mountain plant communities, in the context of climate change.

The impending rising temperatures and reduction in rainfall regimes (Giorgi, 2008; Nogués-Bravo et al., 2008), coupled with the upward shift of the tree line, decrease in plant species richness and overall decline in the frequency of high-mountain specialists reported by Pauli et al. (2012) and Jiménez-Alfaro et al. (2014) in mountains from Southern Europe and Sierra de Guadarrama, respectively; confirms the high vulnerability of Mediterranean high-mountain ecosystems to the effects of global change (Gutiérrez-Girón and Gavilán, 2013). In turn, several scientific endeavors have been undertaken to understand the functional diversity and structure of their plant communities (Escudero et al., 2004; Gavilán et al. 2002; Gavilán et al. 2012; Gutiérrez-

Girón and Gavilán, 2013; Jiménez-Alfaro et al., 2014; Pescador et al., 2014; Pescador et al., 2015); added to those investigating the reproductive strategies in different species and functional groups comprising these Mediterranean high-mountain communities (García-Camacho and Escudero, 2009; Giménez-Benavides et al., 2007; Giménez-Benavides et al., 2011). Nonetheless, studies at the physiological level, expressly those focusing on plant responses to abiotic stress, remain scanty for these Mediterranean high-mountain orophytes (Lara-Romero et al., 2014; Pescador et al., 2016). The exacerbation of the severity gradients in Mediterranean high-mountains, as an outcome of global change, could threaten the rich biodiversity inhabiting these summits through the alteration of plant-plant interactions, and the relationships with biotic and abiotic factors (e.g. herbivory, nutrient availability; Cavieres et al., 2014; Gutiérrez-Girón and Gavilán 2013). Moreover, the growing tendency of more extreme and frequent climatic episodes during the short growing season poses as another pressure on high-mountain species inhabiting the extremes of their altitudinal distribution, particularly in those occupying lower elevations. This could translate into an increased competing pressure with either cohabiting high-mountain species, or with lowland plants feasibly moving up of the tree line and displaying similar or even higher tolerance to the limiting factors (i.e. drought stress, temperatures) and aggravated conditions present at the lower limits of the altitudinal gradient in high-mountain areas. The threat of biodiversity loss due to the aforementioned climate exacerbation and potential increase in species-competition highlights the importance of elucidating the specific physiological mechanisms driving stress responses in high-mountain orophytes from Sierra de Guadarrama; mainly in the receding dry-grassland specialists and narrowly-distributed species.

PLANT FUNCTIONAL TRAITS

Plant traits can determine species differences in productivity and performance, along with aiding in defining species distribution and community assembly (Cornelissen et al., 2003; Garnier and Navas, 2012; Pérez-Harguindeguy et al., 2013). Given their physiological and ecological relevance, implementing plant functional traits (PFTs) enables the identification and classification of ecological strategies of species, at both the individual and group levels. This further relates to environmental conditions and, in turn, allows unveiling the role of individual species in an ecosystem (Lavorel and Garnier, 2002). In addition, study of these PFTs can contribute to the identification of vulnerable and resilient species within ecosystems, in the context of the current climate and biodiversity crises.

In recent decades, scientific efforts have come together to develop a system to define a set of plant functional attributes able to assess, in a comparative and efficient way, plant status and performance at the global scale. As a result, the work by Cornelissen et al. (2003) developed a

complete framework of plant traits that fulfilled these objectives; along with detailed protocols and the ecological, functional relevance for each trait included.

Morphological traits represent the widest and most studied set of PFTs, being foliar traits the most implemented ones to assess plant fitness, since the leaves represent the main organ for resource assimilation in plants. Among morphological foliar traits, the two mainly used are the leaf mass per area (LMA), denoting the ratio between leaf dry mass and leaf area; and its inverse, the more customarily used specific leaf area (SLA) (Cornelissen et al., 2003). Their widespread use relates to their high correlation with key leaf processes (photosynthetic and respiration rates), plant functioning (e.g. growth rate, resource gain and allocation), as well as ecosystem processes (i.e. decomposition rate) (Cornelissen et al., 2003; Garnier et al., 2004). In addition, the LMA can be subdivided, providing information of other leaf traits such as the leaf density (LD) or the leaf volume-to-area ratio (LVA or thickness). Furthermore, leaf area influences the leaf hydraulic balance and leaf thermoregulation, the latter via the boundary layer thickness (Bertolino et al., 2019; Michaletz et al., 2016). Thus, in ecosystems with low productivity where the concomitant action of challenging environmental factors limits resource acquisition, plants generally foster PFTs related to a slow resource gaining and persistence of their structures (Reich et al., 1999). For instance, developing smaller leaves in xeric or water-constrained habitats would allow reducing water loss, added to faster turnover rates and lower resource investment into the growth of new structures (Roth-Nebelsick and Konrad, 2019; Westoby et al., 2002).

As mentioned before, on top of evaluating plant fitness, PFTs can also be implemented to assess the plant performance and its responses to single or multiple abiotic constraints. For instance, using secondary metabolites or measurements of physiological parameters that provide a suitable and accurate indication of stress responses in plants. These can result in a useful tool to assess the intra-specific trait variability along ecological and altitudinal gradients, contributing to comprehend responses of species to global and local environmental drivers. At the plant community level, changes in species abundance and composition, added to the intra-specific functional responses of the component species, are impending among locations throughout altitudinal gradients (Pescador et al., 2015).

Once again, the direct relationship of leaf traits with carbon gain, nutrient loss, and water usage in plants, studying the phytochemicals and physiological responses elicited in them will provide essential indicators to determine plant performance; added to its potential involvement in predicting plant responses and fitness in the context of global change (Gornish and Prather, 2014; Yang et al., 2011).

MATERIALS AND METHODS

STUDY SITE

All the chapters encompassed in the present PhD thesis focus on the study of the vegetation comprised in the xerophytic grassland communities of the Mediterranean high-mountain zones from Sierra de Guadarrama, above the tree line. Expressly, the study plots were situated along the altitudinal gradient of the high-mountain area in the summit Bola del Mundo (1,800-2,244 m), within the Parque Nacional de la Sierra de Guadarrama. Climatic data specific to the study site was obtained from the meteorological station Puerto de Navacerrada (40º 47'35" N, 4º 0'38" O; 1,894 m.a.s.l.).

The Sierra de Guadarrama is a mountainous range of approx. 100 km long, located 70 km northwest of Madrid, Spain. Along with the Sierra de Gredos, Sierra de Béjar, Sierra de Ayllón and Serra d'Estrella, it comprises the Sistema Central, a mountain range with SO-NE direction that divides the two sub-plateaus in the Spanish Peninsula. The environmental conditions experienced are those proper of a Mediterranean-type climate, undergoing harsh, cold winters and extremely dry summers; with an annual mean temperature of 6.4 ºC and mean annual rainfall of 1,350 mm.

The highly dry summer season (May-October) is unique to Mediterranean high-mountain habitats, and it registers less than 10% of the annual rainfall registered in the study area. Furthermore, in areas of Sierra de Guadarrama above 2,150 m.a.s.l., soil water content values fall below 5% and 10% during August and September, respectively (Gutiérrez-Girón and Gavilán, 2013). In turn, snowfall accounts for the main water source in the high-mountain area of Sierra de Guadarrama. Annual variation in snow cover and depth are highly relevant in these summits, due to the inter-annual variability in rainfall patterns in the Mediterranean basin. Concerning the edaphic characteristics, the governing lithology in Sierra de Guadarrama is of a granitic and gneissic nature, conferring the flora a silicic character with limited edaphic variants.

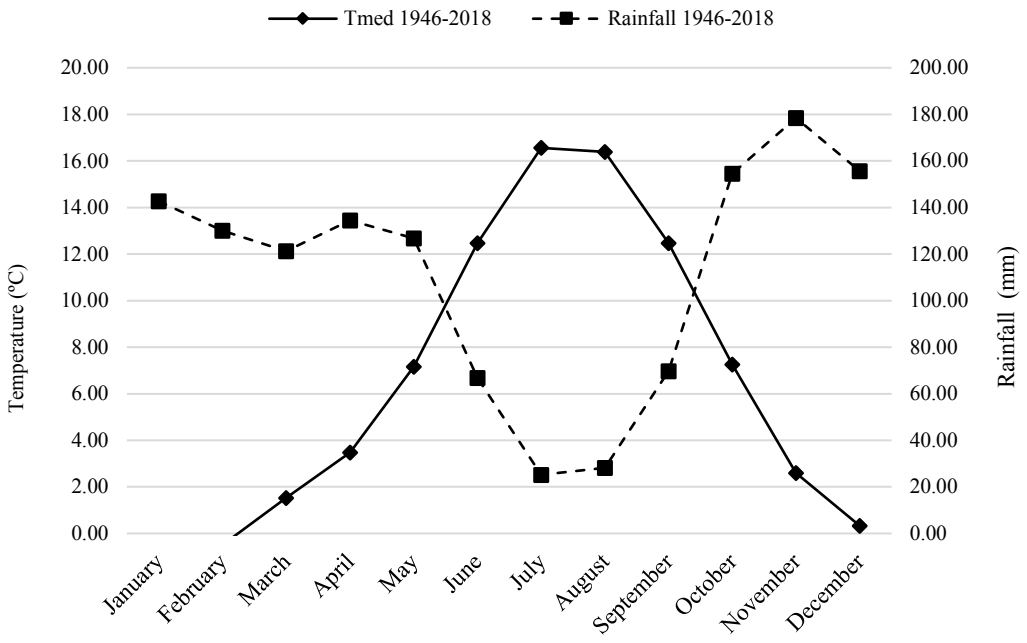

Figure 1. Mean annual temperature and rainfall curves registered in Sierra de Guadarrama. Data from meteorological station Puerto de Navacerrada (1,894 m.a.s.l.), ran by the Spanish Meteorological Agency (AEMET).

Recent studies analyzing climatic parameters from Sierra de Guadarrama have revealed an increasing tendency of warmer temperatures, intensifying in recent decades; encompassing a staggering increase in the mean winter temperature and decreasing snow cover (Muñoz and García-Romero, 2004). In addition, an increasing trend in the incidence of extreme drought episodes has been identified in these mountains since the beginning of the 1990s, together with an abrupt change to overall drier conditions (Ruiz-Labourdette et al., 2014). The findings by Ruiz-Labourdette et al. (2014) also suggest a tendency toward fixed, drier conditions during summer, further constraining the active growing season of vegetation in these areas.

Vegetation above the tree line in Sierra de Guadarrama (1,900–2,000 m.a.s.l.) is dominated by a low dense forest of *Pinus sylvestris* L., replaced upwards by the oromediterranean vegetation belt integrated by an interspersed matrix of shrub species *Cytisus oromediterraneus* and *Juniperus communis* subsp. *alpina*. As altitude increases (from 2,100–2,200 to 2,492 m), the *Cytisus-Juniperus* shrub community is replaced by the patchy xerophytic pasture representative of the cryoromediterranean vegetation belt, dominated by the grass *Festuca curvifolia* Lag. ex Lange, predominantly in the summits and areas free of snowbanks. These plant communities are shaped by the cold temperatures through the year and water scarcity befalling in summertime, fostering the establishment of unique perennial high-mountain specialists, i.e. hemicryptophyte graminoids in association with chamaephytes (prostrate or cushion-form). Vegetation cover varies greatly within these communities and depends greatly on orientation and slopes; having species structure into conspicuous patches or stripes into a bare ground matrix (Gutiérrez Girón and Gavilán, 2010;

Pescador et al., 2014). As mentioned above, *Festuca curvifolia* Lag. ex Lange dominates in these habitats, and it coexists with a number of species, including several species endemic to the Iberian mountains, such as Sierra de Guadarrama (i.e. *Armeria caespitosa* (Gómez Ortega) Boiss. in DC., *Erysimum penyalarense* (Pau) Polatschek, *Hieracium vahlii* Froelich in DC., and *Jasione crispa* (Pourr.) Samp. subsp. *crispa*); coupled with plant species characteristic of the artic-alpine environments (i.e. *Agrostis rupestris* All. and *Phyteuma hemisphaericum* L.)

Table 1. Mean monthly climatic values from meteorological station Puerto de Navacerrada representing the meteorological conditions for the study site (Bola del Mundo). Data refers to the time series 1946-2018. Reference: *Spanish Meteorological Agency (AEMET).*

MONTH	T_{MED}	T_{MAX}	T_{MIN}	$RAIN_{TOTAL}$	$SNOW_{DAYS}$	SUN_{DAYS}	R_0
JANUARY	-0.52	2,19	-3.24	142.68	13	3.32	17
FEBRUARY	-0.50	2.39	-3.38	130.11	13	3.70	15
MARCH	1.52	4.79	-1.74	121.30	11	4.79	18
APRIL	3.47	7.08	-0.15	134.51	10	5.54	16
MAY	7.16	11.19	3.20	126.76	5	7.06	17
JUNE	12.47	17.11	7.79	66.78	1	9.27	21
JULY	16.56	21.85	11.25	25.21	0	11.16	27
AUGUST	16.38	21.63	11.11	28.17	0	10.44	26
SEPTEMBER	12.47	16.94	8.16	69.76	0	7.20	21
OCTOBER	7.25	10.56	3.93	154.54	3	4.81	18
NOVEMBER	2.60	5.36	-0.16	178.42	8	3.36	16
DECEMBER	0.33	2.95	-2.30	155.60	11	3.12	17
ANNUAL	6.60	10.34	2.87	111.15	12	3.32	19

T_{MED}, mean air temperature (ºC); T_{MAX}, Maximum mean air temperature (ºC); T_{MIN}, Minimum mean air temperature (ºC); Rainfall $_{TOTAL}$, mean monthly rainfall including snowfall (mm/month); $Snow_{DAYS}$, average of days with registered snow in a month; Sun_{MEAN}, mean daily insolation per month (hr/day per month); R_0, number of days per month without registered rainfall.

PLANT SPECIES

The environmental variability present within the mountains of Sistema Central is responsible for the great floristic and functional variability observed in the high-mountain plant communities inhabiting these summits (Gutiérrez-Girón and Gavilán, 2013). In this sense, the following assortment of species was selected aiming to incorporate a complete gradient of the vast phylogenetic diversity within these communities (Pescador et al., 2015): *Armeria caespitosa* Ortega) Boiss. in DC., *Erysimum penyalarense* (Pau) Polatschek, *Festuca curvifolia* Lag. ex

Lange, *Juniperus communis* subsp. *alpina, Jurinea humilis* (Desf.) in DC, *Hieracium vahlii* Froelich., and *Senecio pyrenaicus* subsp. *carpetanus* (Willk.) Rivas-Mart.

A brief description of each studied species is given below, in order to provide a wider insight on the diversity and composition of the studied plant community.

Armeria caespitosa (Ortega) Boiss. in DC. (Plumbaginaceae)

Armeria caespitosa is a high-mountain dwarf chamaephyte, endemic to eastern Sistema Central (Sierra de Guadarrama and Sierra de Gredos) in central Spain. It has short branches covered by plentiful foliar debris. It is an early-flowering species, with flowering onset occurring in May-June and blooming soon after snow melts; with flowers taking colors from pink to lilac and grouped in short-scaped flower heads. (Castroviejo et al., 1986)

A. caespitosa appears at altitudes going from 1,700 to 2,430 m, growing in two fused but contrasting habitats: granite cracks and ridges, and dry xerophytic pastures dominated by *Festuca carpetana* (Giménez-Benavides et al., 2011).

Erysimum penyalarense (Pau) Polatschek (Brassicaceae)

Erysimum penyalarense is a perennial herb, endemic to the Sierra de Guadarrama. It presents straight stems (15-25 cm height), lanceolate leaves (basal leaves subspatulated), and big flowers (1-2 cm width), of a bright yellow color. Fruits are in silique, with stigma of two lobes, lasting in the fruit. Anthesis occurs through June and July, followed by fruit development in July and, seldom, in August. (Tutin, 1976)

Its main habitat is the cryoromediterranean grasslands, in the range of 1,950-2,300 m altitude. However, it can also be found underneath oromediterranean bushes (*Cytision oromediterranei*), mainly in north-facing slopes (Fernández-Gonzalez, 1988).

Two main populations have been identified for this endemic of the Guadarrama mountainous system: one in Peñalara peak and another in the Cuerda Larga area (Fernández-González, 1988). Despite the substantial decrease in its distribution range in recent years (i.e. disappearance of population from Peñalara peak), *E. penyalarense* has a relatively constant population with high density of individuals in its remaining locations, with successful yearly re-sprouting and fruit production. However, the high grazing incidence and anthropogenic disturbance (hiking trails, skiing, etc.) threaten the persistence of the species in its main habitat. These factors have been identified as the main cause for the loss of the population in the Peñalara peak before the declaration of the Natural Park in 1985, which additionally reduced the presence of cattle in the

area. In turn, *E. penyalarense* has been cataloged as a threatened species, and included within the Red List for endangered species of the Iberian Peninsula.

Festuca carpetana Fuente, Sánchez-Mata & Rivas Mart. (Poaceae) (=*Festuca curvifolia* Lag. ex Lange, nom. illeg.) (Gramineae)

It is a perennial hemicryptophyte graminoid, commonly found in poor, siliceous substrates. It is a densely tufted perennial grass, with short and bristle-like leaves of a greyish-green color. Flowering occurs in July, with feathery and one-sided panicles.

F. carpetana is the dominant species in the Mediterranean high-mountain xerophytic pastures (>1,900 m.a.s.l.) in the Iberian Peninsula. In Sistema Central, it represents the main structural and cover element of this plant community, forming small ellipsoidal-shaped patches and stripes (up to 30 cm length in bare ground matrix) and cohabiting with other high-mountain specialists (Gavilán et al., 2002; Pescador et al., 2014).

In the upcoming chapters, this species has been referred to with its more traditional name *Festuca curvifolia.*

Hieracium vahlii Froelich in DC. (Asteraceae) (=*Pilosella vahlii* (Froel.) F.W. Sch. & Sch. Bip.)

Hieracium vahlii is an herbaceous species representative of the high-mountain pastures and screes with siliceous substrates. In the Iberian Peninsula, it is found in the Central and Iberic mountainous ranges, as part of the flora representative of oro- and cryoromediterranean pastures (1,700-2,300 m altitude). Leaves, spathulate or oblanceolate, emerge from a basal rosette, and possess long rigid simple hairs. Flowering stems (5-22 cm tall) have numerous stellate, dense, yellowish-stalked hairs often with 1-2 bracts. Flowering occurs in July, with light yellow flowers. (Tutin, 1976)

Its ecology refers to dry high-mountain grasslands, as well as in association with prostrate or broom shrubs inhabiting within the high-mountain habitats. This species gives name to the original description of the dry high-mountain grasslands from Sierra de Guadarrama: *Hieracio myriadeni-Festucetum curvifoliae.*

Juniperus communis subsp. *nana* Syme (Cupressaceae) (=*Juniperus communis* subsp. *alpina* (Suter) Čelak.)

It is an evergreen, prostrate shrub inhabiting subalpine forests and scrublands in direct sun-exposed locations. It has short (3-8 mm), needle-like leaves arranged in whorls of three, with a

single stomatal band on the inner surface. Fruits consist of spherical berry-like cones, which ripen in 18 months.

In the Iberian Peninsula, its main habitat is the oromediterranean scrublands. However, in ancient glacial moraines it descends over to the supramediterranean belt. In the upper limit of the supramediterranean and lower horizon of the oromediterranean belts, it is found with the subsp. *hemisphaerica*; from which it differentiates due to its bearing, and the disposition and morphology of its leaves. (Fernández-González, 1988)

In Sistema Central, it is found intermittently spreading up through high-mountain cryoromediterranean pastures in crests and stony soils, ultimately forming an intercalated matrix with the dominant species, graminoid *F. curvifolia*. In the high-mountain grasslands of Sistema Central, *J. nana* is always in negative associations with most species since it excludes them from its understory, regardless of their strategy (i.e. competitive, pioneer) (Gutiérrez-Girón and Gavilán, 2010). We have used the name published in Euro+Med (2018); however, in most texts throughout this PhD. Thesis we have used the name *J. communis* subsp. *alpina* as shown in Flora Iberica (Castroviejo et al. 1986).

Jurinea humilis (Desf.) DC. (Asteraceae)

It is a perennial herbaceous hemicryptophyte, commonly found in dry, rocky places in mountain ranges and valleys in South-West Europe and Sicilia. It is acaulescent (stems up to 4 cm), with thick rhizomes. Leaves (1.5-4 cm) emerge from a basal rosette, and are pinnatifid, long-petiolated, with arachnoid pubescence. Flowering occurs between June and July, with lilac flowers; followed by fruit development in late July and August. (Tutin, 1976)

In Sierra de Guadarrama, it shows no edaphic preferences and it is mainly orophilic (oro-cryoromediterranean belt), inhabiting xerophytic grasslands. However, it can occasionally appear at lower elevations, between 1,100-1,200 m.a.s.l. (Fernández-González, 1988). It usually denotes an aggregated distribution, which has been linked to positive interactions with other species due to differences in life form, ecological requirements, growing pattern and seed dispersal (Gutiérrez-Girón and Gavilán, 2010).

Senecio pyrenaicus **Loefl. subsp *carpetanus* (Willk.) Rivas-Mart.** (Asteraceae)

Senecio pyrenaicus subsp. *carpetanus* (Willk.) Rivas-Mart. is a perennial hemicryptophyte herb, found in grassy and rocky sites within the high-mountain areas of south-west Europe.

S. pyrenaicus is a very variable perennial herb, with several variants among the Spanish mountainous ranges. *S. pyrenaicus* subsp. *carpetanus* (Willk.) Rivas-Mart. from Sierra de

Guadarrama is found from altitudes ca. 1,600 m; growing 20-45 cm tall, with erect, densely leafy stems that only branch in inflorescence. This subspecies is, generally, strongly glabrous and has more clearly dentate leaves than the other ones. Flowering takes place from July to September, presenting big yellow flowers. (Tutin, 1976)

S. pyrenaicus subsp. *carpetanus* is a frequent species of the pioneer community in stony, siliceous, mobile scree of the highlands in Sierra de Guadarrama. Its coverage surges alongside increased soil consolidation and integrity in any stony soil, fostering the gradual establishment of species proper of the psicroxerophitic grasslands. *S. carpetanus* can also establish near broom-shrubs in established grasslands, in areas not covered by dominant grass *F. curvifolia*. (Fernández-González, 1988)

EXPERIMENTAL DESIGN

The present study was set in Bola del Mundo, along the altitudinal range of the aforementioned high-mountain grassland community (1,980-2,244 m.a.s.l.), dominated by *Festuca curvifolia*. Fieldwork took place during the 2017 and 2018 growing seasons (June-October), two climatically contrasting years in order to counteract the effect of the high variability in the Mediterranean climate on the results. An additional sampling took place in July 2019 (2 clear days) to collect samples from the extremes of the studied altitudinal gradient for the quantification of photoprotective pigments (xanthophylls and lutein), aiming for an inter-annual comparative. Sampling was limited to the Bola del Mundo summit given the presence of a highway along the mountain that enabled transportation of liquid nitrogen up to the high-mountain areas. The latter was of vital importance since the intentioned experiments required the immediate sample freezing with liquid nitrogen to avoid the denaturalization of the studied metabolites; making obligatory the use of a vehicle to transport the gas-container and the samples from the field to the lab facilities as fast and safely as possible.

Four sites were selected to study the potential variation in PFTs in response to environmental stress along an altitudinal gradient (Table 2). Site selection was established based on the Plan Nacional de Ortofotografía Aérea (PNOA; http://www.ign.es/PNOA/) and the framework of the Geographical Information System (ArcGis 10.2 software – ESRI, 2011), in order to avoid dipped areas or snowbank zones, where snow accumulation drives substantial changes in species composition. Site selection took into account all aspects giving rise to environmental heterogeneity in the selected summit: altitude, aspect, slope, and the level of scrub incidence in the area.

Table 2. Geographical details of the four sampling plots the study site in Bola del Mundo.

SAMPLING POINT	ELEVATION (M. A. S. L.)	COORDINATES	ALTITUDINAL DIFFERENCE WITH PREVIOUS POINT
"Pino" (last *P. sylvestris*)	1,980	40º 47' 11.41" N 3º 59' 44.99" W	-
"2do. punto" (Ventisquero de la Estrada)	2,052	40º 46' 58.55" N 3º 59' 37.42" W	70 M
"Telesilla" (Dos Castillas)	2,172	40º 47' 8.55" N 3º 59' 18.18" W	120 M
Bola del Mundo summit	2,244	40º 47 6.99" N 3º 58' 49.82" W	72 M

Plots of 20x20 m were established in each sampling site. In each plot, the most abundant species were identified, resulting in the selection of the following six species from different phylogenetic groups for the study: *Armeria caespitosa, Erysimum penyalarense, Festuca curvifolia, Juniperus communis* subsp. *alpina, Jurinea humilis, Hieracium vahlii,* and *Senecio carpetanus.* All species are present in the two uppermost plots (2,172 and 2,244 m), whereas only *E. penyalarense, F. curvifolia, J. alpina,* and *S. carpetanus* are present throughout the altitudinal gradient selected for these studies (1,980-2,244 m).

CHLOROPHYLL AND PHOTOSYNTHETIC PIGMENT QUANTIFICATION

Chlorophyll *a*, chlorophyll *b* and total carotenoid content were determined according to the protocol by Lichtenthaler (1987), from a whole-pigment extract from green leaf tissue.

Before the extraction, between 200-100 mg fresh weight of the plant material was used per replicate. All samples were freeze-dried, with the subsequent measurement of their respective dry weight. In a new test tube, approximately 5 mg of the freeze-dried tissue were transferred, followed by the addition of 100 µL-distilled water for rehydration of the sample. After 10 min, 8 mL of 96% ethanol were added to the rehydrated samples, followed by 20 s on the vortex. The test tubes were wrapped in aluminum foil and incubated over-night at room temperature in an exhaustion hood. The following morning, samples were vortexed in order to homogenize the solution, and left for 10 min to allow sedimentation of particulates. Samples that appeared turbid were centrifuged at 10,000 x*g* for 5 min to allow proper sedimentation of suspended particles.

Absorbance of the extracts was measured using a UV-Vis spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Kyoto, Japan), at 470.0, 648.6 and 664.2 nm, using 96% ethanol

as blank. Eventually, extracts were measured at 750 nm to correct for impurities. Content of photosynthetic pigment was determined on a DW basis, using the following equations.

$$Chl_a = \frac{(13.36\, A_{664.2} - 5.19 A_{648.6}) * 8.1}{DW}$$

$$Chl_b = \frac{(27.43\, A_{648.6} - 8.12 A_{664.2}) * 8.1}{DW}$$

$$C_{x+c} = \frac{(4.785\, A_{470} + 3.657 A_{664.2} - 12.76 A_{648.6}) * 8.1}{DW}$$

$$Chl_{a/b} = \frac{C_a}{C_b}$$

Where: $A_{648.6}$= Absorbance at 648.6 nm

$A_{664.2}$= Absorbance at 664.2 nm

A_{470}= Absorbance at 470 nm

DW= dry weight of plant tissue extracted (mg)

XANTHOPHYLL-CYCLE PIGMENTS AND LUTEIN QUANTIFICATION

Quantification of xanthophyll pigments and lutein quantification was performed on leaf samples from individuals collected at both ends of the studied altitudinal gradient in Bola del Mundo (2,244 and 1,980 m.a.s.l.). Sample collection took place in July of both 2018 and 2019, on two sunny days with very similar environmental conditions. Leaf samples were collected at 9, 13 and 16 h and immediately frozen in liquid nitrogen, and later stored at -20 ºC until analysis.

For extraction, ca. 100 mg of fresh leaf sample from each herbaceous species were powdered with liquid nitrogen in a cool mortar, then further extracted with 2mL of acetone. To avoid the presence of any traces of acids, 0.5 g/L of calcium carbonate was added to each solution. Next, the homogenate was centrifuged at 0 ºC for 10 min at 13,000 x*g*. Supernatants were collected and deposited in new Falcon tubes. Next, the pellet from each sample was re-suspended in 2mL of acetone for its re-extraction, following the aforementioned procedure. Combined supernatants were adjusted to a final volume of 5mL in new Falcon tubes. Next, the extracts were filtered through a 0.2 µm syringe filter into HPLC vials and stored at -20 ºC until analysis.

Samples were analyzed by reverse-phase HPLC to determine the content of antheraxanthin, violaxanthin, zeaxanthin, and lutein according to the method by García-Plazaola and Becerril (1999). Chromatographic analyses were performed using a Mediterranea Sea18 reverse-phased column (150 x 4.6 mm; 3-µm particle size) (Teknokroma Analitica S.A. Barcelona, Spain). Two mobile phases were implemented, with the following compositions: **A,** acetonitrile: methanol: water (84:9:7); and **B,** methanol: ethyl acetate (68:32). Photosynthetic pigments were eluted using a linear gradient from 100% A to 100% B for 12 min; followed by an isocratic elution with 100%

B for 6 min. Next, a linear gradient from 100% **B** to 100% **A** was applied for 1 min. Finally, an isocratic elution of 100% **A** was used for 6 min to allow the column to re-equilibrate before the next injection. Injection volume was 20 µL, with 1.2 mL/min for solvent flow rate and a working pressure of approx. 82.7 bar. Methanol was flushed into the column for overnight storage.

Samples were scanned with an HPLC-PAD (photodiode array detector) within the 250-450 nm range, with eventual scanning from 250-700 nm for detection of impurities. Peak integration and areas were calculated using Chemstation software (Agilent Technologies, Santa Clara, CA, USA) for Lut and xanthophyll-cycle pigments, whose quantification was performed by comparison with standards. De-epoxidation state of xanthophyll pigment pool was estimated as follows and given in percentage:

$$(Z + 0.5 * A) \Big/ (V + A + Z)$$

Where: A, Antheraxanthin; V, Violaxanthin; Z, Zeaxanthin

PROLINE QUANTIFICATION

The determination of free-proline content in the leaf tissue of the studied species was performed using the ninhydrin-based colorimetric assay as described by Abrahám et al. (2010). This assay was chosen because it is a low-cost, quantitative, reliable and simple method to perform, in addition to it been effectively assessed in numerous plant species (Abrahám et al., 2010).

Fully expanded, healthy-looking leaves from all studied species were selected for this analysis. Three replicates were taken for each species from every sampling point, in order to minimize the statistical error. For each reaction, approximately 100 mg of fresh leaf weight per sample (mg_{FW}) were thoroughly grinded in liquid nitrogen, until obtaining a fine powder.

A 3% sulfosalicylic acid solution was prepared before the analysis, dissolving 3 g of 5-sulfosalicylic acid (2-hydroxy-5-sulfobenzoic acid) in 80 mL distilled water, made up to a final volume of 100 mL. A solution of acidic ninhydrin was also prepared by dissolving 1.25 g ninhydrin (1, 2, 3-indantrione monohydrate) in 30 mL glacial acetic acid and 20 mL of 6 M orthophosporic acid. This solution was dissolved using moderate vortexing and gentle warming. The acidic-ninhydrin solution was kept at room temperature for analysis, and at stored at 4 °C afterwards, up to one week.

First, 3% sulfosalicylic acid was added to the grounded leaf sample (5 µL/mg_{FW}), followed by the thorough grinding of the plant material. The reaction tubes were kept on ice until all the samples were finished. Next, the samples were centrifuged for 5 min at room temperature using

a bench top centrifuge to maximum speed (12,000 x*g*). While the tubes were in the centrifuge, the reaction mixture was prepared by adding 100 µL of 3% sulfosalicylic acid, 200 µL glacial acetic acid, and 200 µL acidic ninhydrin to new tubes. Next, 100 µL from the supernatant of the plant extract were added to the new tubes containing the reaction mixture. The tubes were carefully mixed and incubated at 96 °C for 60 min. The reaction was terminated on ice.

Extraction of the samples was performed by adding 1 mL toluene to each reaction tube, following gentle vortexing for 20 s and left to settle for 10-20 min, until the separation of the organic and water phases could be observed clearly. The chromophore of the organic part, containing the toluene, was then used for measuring absorbance at 520 nm with a UV-Vis spectrophotometer and quartz cuvettes (UV-1700 PharmaSpec, Shimadzu, Kyoto, Japan). Toluene was used as blank. Proline concentration of each sample was determined using a standard concentration curve and calculated on fresh weight basis (µg/g_{FW} or µmol/g_{FW}), as follows.

$$\frac{\mu g \frac{proline}{mL} \; x \; ml_{toluene}}{115.5 \frac{\mu g}{mol}}) / (\frac{g_{sample}}{5})$$

QUANTIFICATION OF NON-STRUCTURAL CARBOHYDRATES

Fully expanded, healthy leaves collected in the field were immediately frozen in liquid nitrogen after excision, to avoid degradation of metabolites. In order to minimize diurnal NSC fluctuations, all samples were collected in a very short period in the morning. Leaf samples collected at the end of each growing season were used to study the effect of altitude on NSC accumulation in the studied species.

For extraction, we followed the protocol by Weiss and Alt (2017) with minor adjustments to fit the DW of the small leaves of the studied orophytes. For each sample, approximately 50 mg of freeze-dried plant material was transferred to 12 mL Falcon tubes and mixed with ultra-pure water (HPLC grade). Tubes containing the samples were placed in a horizontal shaker for 1 h at 30 revolutions/min. Afterwards, 1.5 mL of the supernatant were filtered through an HPLC syringe filter (0.45 µm) and transferred into an HPLC vial. Content of soluble sugars (inulin, fructose, glucose, raffinose and sucrose) was determined via HPLC.

HPLC analyses were performed on a UHPLC Nexera system (Shimadzu, Corp. Kyoto, Japan) equipped with a gradient pump, an auto sampler (storage temperature 4 °C), a column oven and a refraction index detector (RID). Separation of carbohydrates was carried out on a Carbo Sep CHO-682 lead carbohydrate column (300 x 7.8 mm), which was preceded by a Carbo Sep CHO-682 (24 x 2.0 mm) guard kit. Guard column operated at a constant temperature of 85 °C, with a flow rate of 0.4 mL/min, using HPLC-grade water as eluent. Sample injection volume was 20 µL.

Isocratic run was performed for 50 min. Compound identification was obtained by comparing retention times with those from the soluble-sugar standards. Concentration of each soluble sugar ($\mu g/mg_{DW}$) was determined via peak integration from the chromatogram, and the total NSC for each sample was calculated with the sum of all quantified carbohydrates.

STOMATAL FEATURES IN *SENECIO CARPETANUS*

Stomatal features were evaluated on the fully developed leaves of *Senecio pyrenaicus* subsp. *carpetanus* (Willk.) Rivas-Mart., in herbarium specimens collected between 1946 to 2018, within the altitudinal distribution range for this species in the high-mountain areas of Sierra de Guadarrama (above 1,800 m.a.s.l.). In turn, the study area corresponded to the highest summits in Sierra de Guadarrama.

Three leaves were assessed per individual. Leaf imprints were made following the silicon rubber impression technique (Weyers and Johansen, 1985) and image analysis was carried out as described by Fanourakis et al. (2013).

Leaf imprints were made using impression material (A-Silicone Putty Fast Set. VANINI Dental Industry, Italy). Imprints were made in the mid-portion of the leaf on both adaxial and abaxial surfaces, avoiding the zone near the main vein of the leaves. A total of 20 stomata were randomly chosen on each leaf surface, to determine stomatal length and width (5 stomata per field of view). Stomatal density was determined in five non-overlapping rectangular fields of view per imprint.

STATISTICAL ANALYSIS

Diverse statistical methods were applied to the data, selected in order to respond the specific research questions raised in each chapter. Generally, the implemented variables were of the continuous type, deriving in the use of simple linear models, generalized linear models (GLMs) or linear mixed models (LMMs), as seen in Chapters 2 and 4. These methods were used in order to explain the direct relationship between the different continuous variables and their respective explicative variables taking into account random factors, when applicable. Moreover, PCA analysis was implemented in Chapter 3, to find the most relevant climatic parameters within our climate database and the most relevant plant traits within the study data. PCAs were also used to study the correlation between the axis 1 of the climatic variables and the selected plant traits.

All statistical analyses were performed using R program (R Core Team, 2012), and some of its libraries related to GLMs, linear models and other tools for statistical analyses (e.g. FactoMiner, lattice, lme4, MuMin, nlme).

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OBJECTIVES

The main aim of this PhD thesis is to unveil some of the physiological mechanisms driving the life and establishment of high mountain specialists (hemicryptophytes and chamaephytes) representative of the summits from Sierra de Guadarrama, and their potential link to the adaptive capacity of these species to the combining adverse environmental conditions that dictate plant life in high-mountain areas. Previous studies have exposed the complexity and difficulties with the scale when performing field experiments in the study system in question. In order to overcome the potential arising of these obstacles in our field experiments, a multi-scale approach was implemented involving environmental, functional, phylogenetic and spatial schemes. In accordance, the following specific objectives were established:

- Identify some of the specific physiological responses and mechanisms featured in orophytes from Mediterranean high-mountains to counteract the primary constraint for plant performance and survival in these habitats, summer stress, via the assessment of leaf functional traits established as indicators of plant fitness and status in species from other mountainous systems.

- Strive to define some practical plant stress indicators that accurately evaluate the responses, fitness and status of high-mountain flora under the interplaying environmental constraints proper of these habitats.

- Assess the underlying traits in orophytes inhabiting Mediterranean high-mountains, within the context of autecology, to further predict the long-term variations in the composition of Mediterranean high-mountain communities to climatic disturbances; ultimately improving our estimates of the community responses and resilience to climate aggravation.

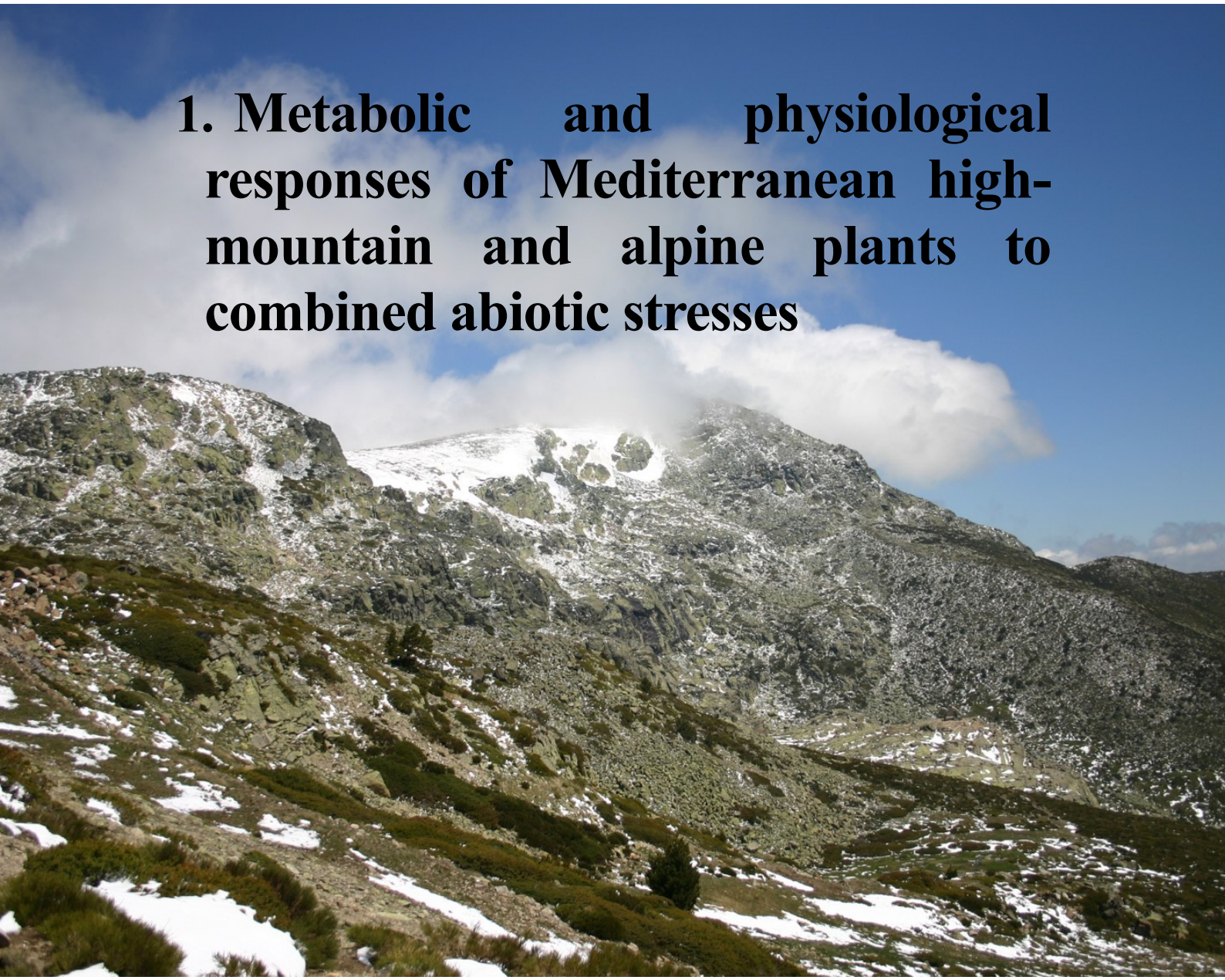
- Evaluate the effect of temporal and altitudinal gradients on the responses elicited by the species to summer stress, aiming to reveal if these responses are stress-mediated (induced response varying according to the severity of the stress) or constitutive (elicited whether the plant is under stress or not).

- Study the role and extent of the potential involvement of osmolytes proline and non-structural carbohydrates in the stress-response of Mediterranean high-mountain orophytes to both the seasonal heightening of summer drought and the drought-severity gradient, the latter inversely correlated with altitude.

- Uncover the influence of long-term variations in the climatic regimes in these summits on the potential variation in morpho- and micro-morphological leaf traits (leaf area, leaf width, stomatal density and size) in a frequent species of the pioneer community of the stony, siliceous uplands of Sierra de Guadarrama, to explore its potential adaptive capacity to the impending climate exacerbation in these summits.

- Provide a preliminary assessment of the role of photoprotective pigments and strategies in Mediterranean high-mountain specialists to the wide thermal amplitude, co-occurring with high irradiance and drought in the brief period of optimum plant activity, between contrasting altitudes and growing seasons.

- Determine the potential vulnerability and the adaptive capacity of Mediterranean high-mountain plants and, by extension that of Mediterranean high-mountain plant communities, to the foreseen rising temperatures and decline in rainfall regimes outcome of climate change.



1. Metabolic and physiological responses of Mediterranean high-mountain and alpine plants to combined abiotic stresses

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ABSTRACT

High-mountain areas provide excellent opportunities to study the effects of combined abiotic stresses on plant physiology given their variety of steep ecological gradients, low anthropogenic disturbance and remarkable levels of taxonomic diversity. Efficient photoprotective and antioxidant scavenging mechanisms are vital for survival in high-mountain plants, having its altitudinal and seasonal variations determined by environmental or ontogenetic factors such as the decrease in mean temperatures and water availability. A number of stress indicators have been described in order to rapidly assess plant fitness in high-mountain environments. For instance, carbon isotope ($\delta^{13}C$) and proline content as drought and temperature stress indicators, because of their link to water-use efficiency and osmotic adjustment; photosynthetic pigments, related to phenology, nutrient status, light and temperature stress; and non-structural carbohydrate accumulation in response to mild or brief drought conditions. The present review unveils the wide research opportunities available for the study of adaptive responses in high-mountain plants via stress indicators, and calls attention to the substantial knowledge gap existing between alpine zones and other mountainous regions, such as Mediterranean high-mountains. The aim is to grant a more holistic understanding of the physiological mechanisms driving plant life in high altitudes and improve the predictions of the effects of changing environments in these species and across ecological scales.

Abbreviations – Chl, chlorophyll; Chl *a/b*, Chl *a*/Chl *b* ratio; F_v/F_m, maximal photochemical efficiency of photosystem II; LNC, leaf nitrogen concentration; NPQ, non-photochemical quenching; NSC, non-structural carbohydrates; PFTs, plant functional traits; Pro, proline; PSII, photosystem II; ROS, reactive oxygen species.

INTRODUCTION

Simultaneous action of different environmental gradients capable of severely limiting plant performance constitutes one of the main drivers for diversity and plant growth in many habitats, particularly in very stressful ones such as high-mountain areas. This simultaneous action of several stressors acts as a filter, allowing only well-adapted species to thrive (Körner 1999). Altitude influences a wide variety of environmental factors that affect plant performance such as extreme temperatures, precipitation, duration of snow cover, radiation intensity, wind speed and also topography and microclimate (Körner 1999, Winkler et al. 2016). Given the variety of steep ecological gradients, low anthropogenic disturbance and their remarkable levels of taxonomic diversity, high-mountain areas result in ideal models to study the ecological effects of global change on plant physiology (Körner 1999).

This work is a state-of-the-art review on the physiological and adaptive responses in plants from high-altitude habitats, taking into consideration the differences between the two most prominent high-mountain environments in the region: the genuine alpine zones, and the Mediterranean high-mountains (Fig. 1). Alpine environments refer only to temperate-zone areas above the tree line, with reduced air pressure and atmospheric temperature as key components of its macroclimate, added to high solar radiation and without water limitation through the summer season. In this review, the term 'alpine' alludes exclusively to the described alpine environments. In addition, these areas experience annual mean air temperatures of approximately 8.5 ºC in continental areas and approximately 4–5 ºC in coastal or island mountains. Small altitude variations translate into substantial humidity and temperature differences. The growing season in alpine areas occurs during summer, with a mean length and photoperiod between 70–100 days and 15–16 hd−1, respectively (Körner 1999).

Mediterranean high-mountain areas differ from alpine zones and other mountains given the drought incident in summertime (Cavieres et al. 2000, Gutiérrez-Girón and Gavilán 2013). This implies that plant life establishment and performance in Mediterranean high-mountain areas are driven by two severity gradients: (1) low temperature stress rising with altitude, and (2) drought stress declining with altitude (Pescador et al. 2015). In this review, the Mediterranean high-mountain zones comprise the European Mediterranean high-mountains and the Mediterranean Chilean Andes, both having the distinctive intense summer drought befall the growing season. The annual mean temperature is 6.4 and 9.8 ºC, respectively, added to high inter-annual rainfall variability mainly occurring in winter as snow (Sierra-Almeida et al. 2010, Pescador et al. 2016, Gavilán et al. 2018). Microhabitat variations in these regions, induced by local topography at fine scales, can influence small-scale changes in the effective growing season, due to differences in solar radiation incidence or duration of snow cover (Pescador et al. 2016).

The combination of increasing climatic stresses that dictate plant life in high-mountain environments provides a diversity of microclimates within small scales through an altitudinal gradient, mimicking macroclimatic interactions. Plant responses to combined abiotic stressors can be evaluated through plant functional traits (PFTs), given the connection between PFTs and severe environmental events related to climate change (Lloret et al. 2016). The extension of the high-mountain areas above the tree line, along an altitudinal gradient, enables assessing intra- and inter-specific variations in PFTs within and between sites. Among PFTs, morphological traits such as plant height, specific leaf area index, leaf dry matter content, leaf carbon concentration and leaf nitrogen concentration (LNC) have been widely studied in both alpine and Mediterranean high-mountains (see Sierra-Almeida et al. 2009, 2010, Yang et al. 2011, Pescador et al. 2015, Giménez-Benavides et al. 2018). Conversely, little research efforts have been made to study physiological traits in Mediterranean high-mountain species (but see Bernal et al. 2013, Hernández-Fuentes et al. 2015, García-Lino et al. 2017), compared with the numerous studies existing in alpine plants (Streb et al. 2003, Spitaler et al. 2008, Dierking and Kallenbach 2012, Abeli et al. 2015, Buchner et al. 2015). Given that leaf traits are directly related to carbon gain, nutrient loss and water usage in plants, their study could allow determining and predicting plant performance in high-mountain habitats in the context of global change (Yang et al. 2011, Gornish and Prather 2014, Gutiérrez-Girón et al. 2015).

Climate warming, a main driver of global change, can result in adjustments in the competition balance between life forms, through the modification of their fitness due to their phenological sensitivity to temperature. In particular, the predicted changes in temperature and rainfall regimes in high-mountain areas, coupled to the upward shift of the tree line, underline their vulnerability to climate change (Giorgi and Lionello 2008, Gutiérrez-Girón and Gavilán 2013). Moreover, changes in the competition and facilitation mechanisms of plants and the maintenance of high photosynthetic capacity can lead to permanent changes in ecosystem assembly and function (Cavieres et al. 2014, Shi et al. 2014). Studying the variations in physiological traits in high-mountain areas will enable identifying attributes vital for conferring resilience to extreme events, consequence of climate change. In this manner, implementing a series of stress indicators based on the aforementioned attributes poses as a valuable tool to monitor the variation in stress responses among species under simultaneous abiotic pressures. In addition, the use of stress indicators will grant an accurate assessment of plant status and adaptation in high-mountain environments, allowing a better understanding of the interplay of abiotic factors on plant fitness in these habitats.

Meta-analyses have been performed to identify foliar traits that can predict the effects of changing environments in species from low elevations (see Gornish and Prather 2014). However, and to the best of our knowledge, efforts to pinpoint foliar functional traits suitable for accurately

assessing the responses to interplaying environmental constraints in alpine and Mediterranean high-mountain species have not been made yet. The present work reviews the information available up to date on foliar functional traits linked to plant adaptation strategies in response to the combining abiotic factors limiting high-mountain vegetation. In turn, it seeks to endorse the use of practical plant stress indicators suitable for evaluating the adaptive responses of plants inhabiting high-mountain environments. In addition, this review aims to unveil research opportunities towards understanding the physiological mechanisms driving plant life in high-mountain environments, and further safeguard high-mountain flora via abiotic stress indicators for a more efficient assessment of plant fitness in stressful environments.

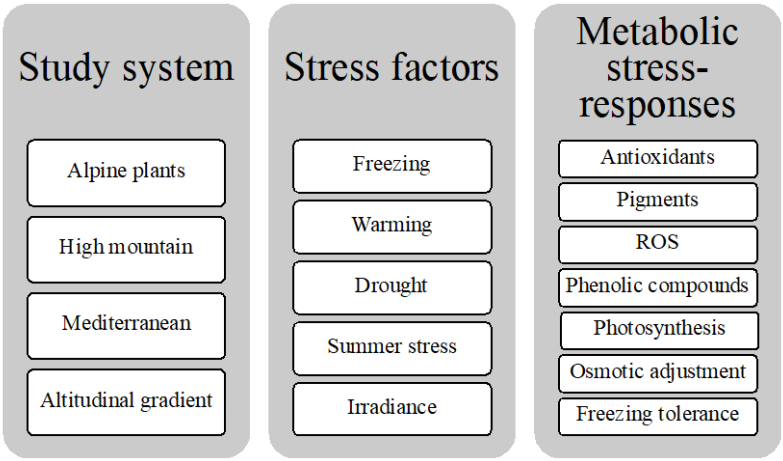

Figure 1. Main subjects enclosed in the revised literature, classified into the three main points of the present review.

ADAPTATION RESPONSES TO ABIOTIC STRESSES IN HIGH-MOUNTAIN PLANTS

Temperature stress

In high-mountain environments, freezing temperatures constitute one of the main adaptive drivers for plant life. Owing to the imminent risk of injury in organs and tissues by low temperatures during the growing season (Ladining et al. 2013), high-mountain plants have adopted several physiological mechanisms to withstand low temperatures, such as accumulation of compatible solutes [i.e. low-molecular weight carbohydrates and proline (Pro)], enhanced phytochemical biosynthesis or modifications to their photosynthetic apparatus. Snow cover, plant-to-plant positive interactions with neighbors (i.e. facilitation), and microclimate heterogeneity can also influence freezing tolerance in alpine species (Venn et al. 2013, Briceño et al. 2014). In any case, the effects diverge among life forms, phenology and geographical location. Low temperatures, early spring halting events, snowmelt timing and exposure play a key role in the distribution of

high-mountain plants. Nonetheless, while these have been extensively studied in alpine flora, scarce studies exist focusing on their influence in Mediterranean high-mountain species (but see Sierra-Almeida et al. 2009, 2010, 2016, Pescador et al. 2016).

Coping with subzero temperatures can be achieved via two mechanisms: avoidance and tolerance. Freezing avoidance refers to the impediment of ice formation within cells (nucleation), which many plants accomplish through either osmotic adjustment or by transient 'super-cooling' (Körner 1999). In contrast, freezing tolerance refers to the ability to endure ice nucleation in extracellular spaces, via protoplast dehydration caused by rapid transfer of water to a growing body of ice, to reduce the intracellular freezing point and increase freezing tolerance (Körner 1999).

Freezing tolerance is co-determined by elevation (Sierra-Almeida et al. 2009, Pescador et al. 2016), and several studies have pointed out freezing tolerance as the prime response mechanism in leaves from alpine species under low temperature stress (Taschler and Neuner 2004, Ladining et al. 2013). Similarly, in the Mediterranean Chilean Andes, the findings by Sierra-Almeida et al. (2009, 2010) identified tolerance as the predominant mechanism to endure low temperatures in most of the studied species. This contrasts with the findings in perennial herbs and European Mediterranean high-mountains, where freezing avoidance has been proposed as the main low temperature-enduring mechanism (Pescador et al. 2016). Nevertheless, species from these contrasting high-mountain environments have shown a similar ability to shift between freezing withstanding mechanisms through the growing season, to provide enhanced protection during late summer frosts (Sierra-Almeida et al. 2009, 2016, Sklenár 2017). The latter suggests the existence of similar selective pressures shaping freezing resistance during the growing season in high-mountain environments (Sklenár 2017).

Low temperature photoinhibition also poses as an impending selective pressure in alpine environments, resulting in photosynthesis being a well-adapted process in high-mountain plants, with certain degree of plasticity. This is shown in the substantial recovery of depressed chlorophyll fluorescence (F_v/F_m) and photosynthetic CO_2 uptake within a short period following low temperature stress (Shi et al. 2004, Bascuñán-Godoy et al. 2010). The latter can be explained as an efficient diurnal regulation of the photosystem II (PSII), related to the harmless dissipation of excess light captured, also known as non-photochemical quenching (NPQ); as well as smaller chloroplast and antenna sizes, optimizing light energy capture (Germino and Smith 2000, Loik et al. 2004). Although such adaptation strategies have been well documented in alpine species (Germino and Smith 2000, Loik et al. 2004, Briceño et al. 2014), studies in their Mediterranean counterparts mainly revise the strategies modulated by low temperature exposure in response to higher excitation pressure of PSII in high-mountain shrubs. For example, the accumulation of

photoprotective pigments and higher Chlorophyll *a/b* ratio (Chl *a/b*) in Mediterranean high-mountain species as an additional light acclimation strategy (Bascuñán-Godoy et al. 2010, Bernal et al. 2013).

In view of the ongoing warming of high-mountain environments and its exacerbation in forthcoming years as a consequence of climate change, there has been an increasing interest to study the responses and potential adaptations of species from these habitats to rising temperatures. Successful experiments in alpine species *Kobresia pygmaea* showed potential for accelerated growth and high resource efficiency use, indicating its inherent advanced physiological capacity and its ability to acclimate to a warmer environment (Yang et al. 2011). In contrast, warming could highly constrain plant physiology in Mediterranean high-mountain species, given the negative impact summer stress poses over the reproductive performance, seed establishment and germination in late-flowering species (Giménez-Benavides et al. 2018).

Temperatures above mean values could also play a modulating role during senescence in alpine perennials by delaying chlorophyll (Chl) degradation and maintaining high LNC, as observed in alpine *Delphinium caeruleum* and *Elymus nutans* following an in situ 'autumn-warming' treatment (Shi et al. 2014). This indicates a higher adaptation capacity of perennial herbs to shifts in temperature, in comparison to non-perennial species (Shi et al. 2014). Furthermore, Buchner et al. (2015) have reported the significant effect of rising temperatures on photosynthetic parameters in *Senecio incanus, Ranunculus glacialis* and *Rhododendron ferrugineum*. The F_v/F_m showed no significant decrease under high temperature in the two species, although a considerable reduction of the values for this parameter was observed in *Rh. ferrugineum*. Nonetheless, complete F_v/F_m recovery was observed for all species by the end of the warming treatment (Buchner et al. 2015). These results are in agreement with the one reported by Shi et al. (2014) and allow inferring that the increase in Chl content under warmer climate could translate into an enhancement of photosynthetic activity, plant growth and carbon assimilation in alpine species, if there is no water limitation. In addition, in situ warming experiments in alpine species suggest warming can also contribute to improving photosynthetic performance, given the higher photosynthetic capacity and photosynthetic nitrogen-use efficiency displayed in alpine plants under experimental warming without water constraints (Yang et al. 2011).

Nevertheless, the negative effects of warmer conditions in mountain areas, such as inducing early snowmelt and cold de-acclimation in alpine species, should not be overlooked. Several *in situ* experiments have reported the influence of warming and variation in snowmelt timing on the freezing tolerance in alpine plants (Loik et al. 2004, Bannister et al. 2005, Briceño et al. 2014). These resulted in a reduced frost tolerance in species inhabiting snowbanks, in comparison to those from snow-free areas, given the thermal insulation provided by snow cover which reduces

the daily leaf temperature amplitude and the number of freeze–thaw cycles these plants are exposed to (Bannister et al. 2005, Briceño et al. 2014). This emphasizes the importance of preserving the rich environmental heterogeneity in alpine areas: existence of ecotypes with a higher frost tolerance in adjacent habitats with less persistent snow cover could safeguard against freeze-induced local extinction as climate warming progresses (Briceño et al. 2014).

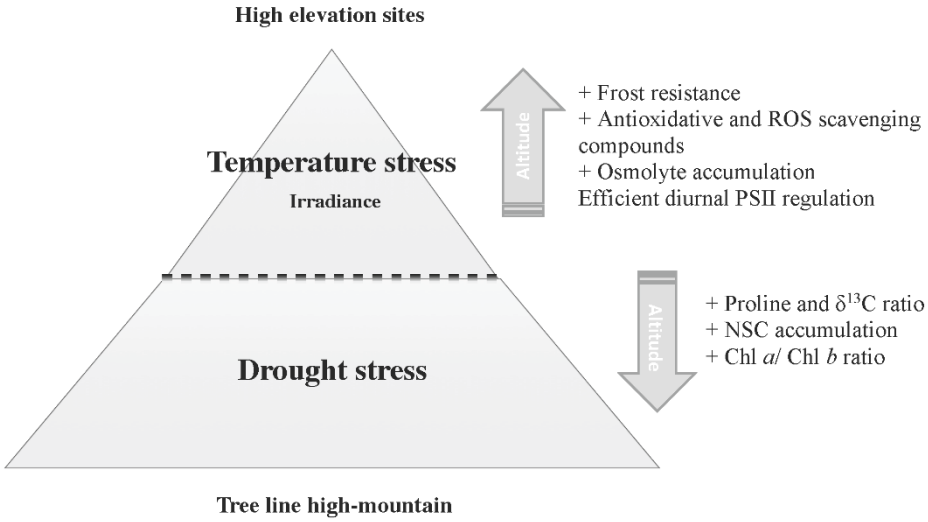

Figure 2. Depiction of the main abiotic stress factors at high (temperature and irradiance) and low (drought) elevations in high-mountain areas.

Mediterranean summer stress

In Mediterranean environments, higher temperatures implicate reduced rainfall and higher frequency of extreme climatic events, coarsening the conditions for species to thrive. Contrasting to the favoring effects of climate warming for alpine plants through the extension of the active growing season, the major outcome of climate warming for the Mediterranean high-mountain plants will be the lengthening of the summer stress period (Giorgi and Lionello 2008). The latter will elicit a series of adaptive responses in plants from this habitat, entailing shifts or anticipation of specific developmental stages (e.g. early flowering onset; Giménez-Benavides et al. 2011).

Summer stress is regarded as the main constraint for plant performance and survival in Mediterranean regions, and comprises the simultaneous effect of elevated temperatures, high irradiance and water scarcity (Larcher 2000). Plants from Mediterranean high-mountain habitats have developed different photoprotective strategies and biochemical adaptations to avoid detrimental effects, outcome of such adverse conditions (Aragón et al. 2008, Bascuñán-Godoy et al. 2010). The reversible decrease in the photochemical efficiency of PSII at midday during summer is an additional example of the aforementioned shielding strategies, suggesting the

presence of highly efficient photoprotection mechanisms to avoid overheating and photoinhibition in Mediterranean high-mountain species (Aragón et al. 2008).

In Mediterranean environments, summer drought is considered a primary constraint for biological activity (Gutiérrez-Girón et al. 2015, Giménez-Benavides et al. 2018). The severe decrease in soil water potential, outcome of summer stress, leads to declines in photosynthesis and increases in photodamage in these habitats (Hernández-Fuentes et al. 2015). Hence, elevated temperatures combined with drought stress can induce diffusional and biochemical photosynthetic limitations in high-mountain species from lower ends of the elevation gradient, as observed in perennial herbs from the Chilean Andes (Sanfuentes et al. 2012, Hernández-Fuentes et al. 2015). In contrast, growth and metabolism display no limitation by warming at higher altitudes, where warming and water limitation decrease. In fact, warming positively influenced photosynthetic performance in *Phacelia secunda* at high elevations (Sanfuentes et al. 2012, Hernández-Fuentes et al. 2015), demonstrating that the photoprotective and photosynthetic responses of high-mountain species to warming is dependent on both elevation and soil moisture availability (Hernández-Fuentes et al. 2015), as presented in Fig. 2. Additionally, summer stress could promote negative effects on the reproductive performance, seed germination and establishment of Mediterranean high-mountain vegetation (see Giménez-Benavides et al. 2018). However, the degree of susceptibility may be species-specific and related to their phenological strategy, adaptability to climatic conditions and microhabitat (Larcher 2000, Giménez-Benavides et al. 2018). The latter has been observed in late-flowering *Silene ciliata*, where extreme hot and dry weather caused severe reproductive failure at the lowest distribution limit, contrasting with the stable reproductive performance of early-flowering high-mountain specialist *Armeria caespitosa* (García-Camacho and Escudero 2009, Giménez- Benavides et al. 2018). Potential adjustment of plant growth, flowering phenology and resource allocation have been proposed as a dynamic local adaptation mechanism to withstand drought stress in cushion and shrubs along the elevation gradient, driven by a critical temperature threshold rather than photoperiod (Aragón et al. 2008, García-Camacho and Escudero 2009).

Nonetheless, it is important to note that other factors may influence shifts in reproductive performance in these species. For example, alterations in plant–pollinator interactions (Giménez-Benavides et al. 2018) and the upward shift of the tree line, shrinking the space for high-mountain flora to germinate and grow (Körner 1999).

STRESS INDICATORS: EVALUATING ADAPTATION RESPONSES TO ABIOTIC STRESSES

Monitoring plant fitness and other functional features that allow adaptation and survival of plants in high-mountain environments has contributed to proposing a number of potential physiological stress indicators. These can be very useful and valuable for different questions, such as evaluating the effects of extreme temperatures, drought and irradiance or anthropogenic disturbance on plant fitness (Corcuera et al. 2012, Wen and Chen 2014).

Osmolytes and carbon isotope as abiotic stress indicators

There is a clear convergence between responses to drought and to low temperature stress. Osmotic adjustment is a key response mechanism in plants under water deficit and low temperatures, achieved through the accumulation of compatible solutes in the protoplasm [i.e. amino acids and non-structural carbohydrates (NSC)].

For instance, Pro accumulation has been identified as an indicator of physiological disturbance in crops under salinity, drought or extreme temperature stress (Hayat et al. 2012). In addition, its role in stabilizing the mitochondrial electron transport system under stress conditions suggests a direct link between Pro buildup and enhanced photosynthetic yield (Hayat et al. 2012). Similarly, NSC accumulation and allocation ratios in plant organs have been regarded as physiological attributes to assess carbon costs and adaptation strategies in plants, due to the contribution of NSC in osmotic adjustment and signaling processes enabling physiological adaptations to changing environments (Körner 1999).

Carbon isotope ($\delta^{13}C$) and Pro content have been proposed as drought and temperature stress indicators in different plant species, given their link to water-use efficiency and osmotic adjustment, respectively (Farquhar et al. 1989, Corcuera et al. 2012, Hayat et al. 2012). In alpine species, studies in juniper needles reported a significant negative correlation among $\delta^{13}C$ and Pro accumulation with soil water content and rainfall (Wen and Chen 2014). In turn, the higher $\delta^{13}C$ and Pro content in these plants was indicative of the physiological response to drought (Wen and Chen 2014). Additionally, high Pro content has also been found in *Pinus pinaster* populations from xeric sites in response to drought stress (Corcuera et al. 2012).

Substantial altitudinal increases in Pro concentrations have been reported in herbaceous alpine species, outcome of joint decreasing temperatures and inadequate water conditions. A comparison among several high-mountain *Festuca arundinacea* genotypes showed how Pro accumulation drastically increases in alpine accessions in response to cold stress, and the relationship between higher Pro accumulation and superior freezing tolerance in these accessions (Dierking and Kallenbach 2012). The latter provides further proof that Pro accumulation would be a proficient plant stress indicator for high-mountain species and, therefore, should be included in studies for

an accurate, easy and prompt assessment of plant status under drought and temperature stress in high-mountain areas.

In Mediterranean high-mountain species, besides the customary whole-plant and leaf traits, researchers have focused on studying PFTs associated to resource acquisition and nutrient cycling because of their link to PFTs that are more orthodox. Such is the case for $\delta^{13}C$, related to water-use efficiency, drought tolerance and photosynthetic capacity and nitrogen isotope ($\delta^{15}N$), associated with multiple N-related processes (Farquhar et al. 1989, Pescador et al. 2015). However, novel research efforts need to be made to foster the study of metabolic stress indicators to evaluate plant fitness and adaptation in Mediterranean environments. For example, the work by García-Lino et al. (2017), using NSC accumulation to assess the effect of mild drought stress in Mediterranean high-mountain cushion plants, provided further evidence that photosynthetic carbon assimilation follows the sink limitation hypothesis as altitude increases. More compact shapes observed in cushions from the upper- most populations are related to a decrease in NSC concentration. This pattern is markedly different towards the dry, lower end of the altitudinal gradient, where a lack of significant changes in size and NSC concentration was observed (García-Lino et al. 2017). The latter highlights the higher adaptation strategies of high-altitudes plants against the complex interplay of stressful conditions, compared with their counterparts from lower elevations.

Moreover, NSC accumulation has also been reported as a physiological mechanism potentially linked to the freezing-drought response in high-mountain plants from the Chilean Andes. The enhanced frost tolerance observed in *Chuquiraga oppositifolia* and *Taraxacum officinale* under drought stress, compared with those under irrigation treatment, could be attributed to an osmotic adjustment provided by higher NSC concentrations in these species in response to drought stress, delaying ice crystal formation and intercellular halting damage (Sierra-Almeida et al. 2016).

Phytochemicals and antioxidant enzymes as abiotic stress indicators

Photosynthesis is one of the most temperature-sensitive physiological processes in plants, with thermolabile components in both dark and light reactions. Multiple abiotic stresses enhance photosynthetic generation and buildup of reactive oxygen species (ROS), which could damage the repair mechanisms of PSII resulting in reduced photosynthesis and, thus, a potential reduction in carbon fixation (Streb et al. 2003, Peñuelas and Munné-Bosch 2005). Hence, several adaptation mechanisms and crosstalk between abiotic stress signaling are triggered in plants to mitigate its effects on the photosynthetic electron transport (Peñuelas and Munné-Bosch 2005).

Efficient ROS scavenging mechanisms can be imperative for survival in alpine species (Körner 1999). Highly efficient photoprotection has been proposed in high-mountain plants through different sources of ROS scavenging compounds such as the antioxidant enzymes superoxide

dismutase, catalase and ascorbate peroxidase in combination with other ROS detoxifying metabolites, i.e. phenolic compounds (Peñuelas and Munné-Bosch 2005). Field studies in alpine herbaceous plants exhibit a pronounced altitudinal variation in the content of phenolic compounds and antioxidant/Chl ratio, confirming the assorted and superior antioxidant capacity of plant species from higher altitudes in response to higher solar irradiance in these areas, compared with the ones from lower elevations (Körner 1999, Spitaler et al. 2008, Zidorn 2010).

Studies in alpine plants have depicted a highly efficient ROS scavenging capacity under combined drought and heat stress, suggesting their great adaptive response to warming. The significant increases in de-epoxidation of the xanthophyll cycle in alpine species under heat stress indicate its important role as a photoprotection strategy, enhancing the stability of the photosynthetic apparatus under elevated temperatures through NPQ and the stabilization of the thylakoid membrane and PSII reaction centers (Buchner et al. 2015).

Assessment of photosynthetic pigment composition can be employed as an indicator of plant physiological status. It can provide insight on the phenology, nutrient status and general stress through quantification of Chl *a* and carotenoids, together with offering information about the photosynthetic efficiency and dissipation of excess energy via the Chl *a/b* ratio or assessment of the oxidation status of the xanthophyll cycle, respectively (Spitaler et al. 2008, Zidorn 2010). Moreover, measuring the ratio of F_v/F_m can be a reliable and rapid indication of changes in the maximum photochemical efficiency of PSII, allowing monitoring the effects of temperature stress or water deficit in plants (Ritchie 2006).

Pigment analysis, together with F_v/F_m measurements, can utterly provide a fast indication of the level of damage and predict the subsequent performance of plants under both temperature and drought stress, as measurements can be performed instantly after a stress event (Ritchie 2006). Total Chl content and Chl *a/b* ratio are highly sensitive to the light environment and water stress, making them good indicators of oxidative stress in plant tissues. Nonetheless, the hierarchical effect of environmental factors is to be considered, since it plays a fundamental role in the response of plants to combined stressors (de Bello et al. 2013). For instance, water availability has a predominant role over increased photoperiod in different populations of *Silene suecica*, validated by the responses in functional traits related to water stress, such as a decreased total Chl content and higher Chl *a*/*b* ratio (Abeli et al. 2015). In herbaceous alpine species, the decrease in mean temperatures with altitude is the key factor driving the significant accumulation of antioxidants and ROS scavenging compounds in leaves, and not the higher UV-B irradiance as generally assumed (Streb et al. 2003, Spitaler et al. 2008, Albert et al. 2009, Bernal et al. 2013). Studies in *Ra. glacialis* and *Arnica montana* grown in high UV-B radiation conditions showed how only the decrease of 5 °C and the de-acclimation to the alpine environment have a significant

effect on shifting the phenolic composition in these species (Streb et al. 2003, Spitaler et al. 2008, Albert et al. 2009). In addition, the study by Buchner et al. (2015) in alpine plants under heat stress in light and dark conditions reported only a significant increase of ROS scavenging activity under dark treatment. The more damaging effect of heat treatment under dark conditions, compared with the one in the light environment, ratifies the hierarchical effect of temperature stress over irradiance in alpine species. However, their interaction remains a driving factor influencing plant life in high mountains.

Compared with the extensive studies on altitudinal variations of phytochemicals and secondary metabolites in alpine species in response to combined abiotic factors (Streb et al. 2003, Shi et al. 2004, Spitaler et al. 2008, Albert et al. 2009, Buchner et al. 2015), studies in Mediterranean high-mountains mostly concern shrubs and woody species (Bernal et al. 2013, Nenadis et al. 2015), while studies regarding herbaceous species remain scant.

Even at high elevations, UV radiation is not the main factor driving phenolic and flavonoids accumulation in Mediterranean high-mountain shrubs. Results from UV-exclusion treatment in *Buxus sempervirens* leaves revealed that UV irradiance has a minor hierarchical stress effect in plants from Mediterranean environments (Bernal et al. 2013). In accordance, the study by Nenadis et al. (2015) indicates that UV induced no effect on the total phenolic content during the growing season in *Arbutus unedo*. Thus, other environmental or ontogenetic factors could be the main triggers for altitudinal and seasonal variations of phenolic contents. For instance, response to low temperature stress at higher elevations or during autumn and winter or required protection against UV-B in early developmental stages due to less developed cuticles and cell walls, granting protection to combined adverse factors (Bernal et al. 2013, Nenadis et al. 2015).

The triggering of alternative electron sinks under the effect of severe drought stress has allowed disregarding photoprotective mechanisms of thermal dissipation, i.e. xanthophyll cycle, as the main approach to cope with the excess energy absorbed in the event of concurrent warming and severe drought stress (Hernández-Fuentes et al. 2015). The latter exacerbates the complexity behind physiological plant responses to climatic extremes, and advocates combining indicators that remain unvaried under combined stress incidence (i.e. Pro buildup, $\delta^{13}C$ and $\delta^{15}N$) with those more sensitive to extreme climatic events, such as photosynthetic pigments.

CONCLUSIONS

Plant life in high-mountain areas is driven by a combination of abiotic stressors, extreme temperatures and drought stress being the most significant for plant fitness and establishment in alpine and Mediterranean high-mountains, respectively. These interplays of factors prompt a

series of physiological and phenological adaptation strategies in plants to counteract their effect throughout the growing season.

Extensive studies on the responses to combined abiotic constraints in alpine flora have demonstrated the convenience of using $\delta^{13}C$, F_v/F_m, NSC accumulation, phytochemicals and Pro buildup as indicators of plant physiological status, providing insight on the phenology, nutrient status and general plant stress under drought, temperature and irradiance stress. However, scarce information is available in their Mediterranean counterparts. This lack of information opens a wide field of opportunities for future eco-physiological studies in Mediterranean high-mountain vegetation, centered on stress indicators proven efficient to assess the stress level of alpine plants subjected to summer stress, which is the main constraint for plant life in Mediterranean high-mountains. The latter is due to the similar responses observed in plants from alpine areas and the Mediterranean Chilean Andes, such as switching between freezing withstanding mechanisms, which allows inferring resembling stress responses given the similar combination of extreme abiotic pressures in these divergent high-mountain habitats.

The study of these indicators will help closing the profound knowledge gap between alpine and Mediterranean high-mountain vegetation. Moreover, it will allow comparing between plant responses among regions, along with the designation of more accurate stress indicators for assessing fitness in each high-mountain environment. Greater research endeavors are needed regarding other factors shaping plant life (i.e. effects of snow cover, recruitment and plant–plant interactions or the linkage between root traits and abiotic and biotic factors) to grant a more holistic understanding of the physiological mechanisms driving plant life in challenging environments. In turn, this will enhance our ability to predict the responses of plant species to more challenging environmental conditions, outcome of global change.

Author contributions

R.M.U., A.E. and R.G.G. conceived and designed the outline and content of the article; R.M.U. wrote the manuscript with supervision by A.E.; R.M.U., A.E. and R.G.G. revised bibliography and provided editorial advice.

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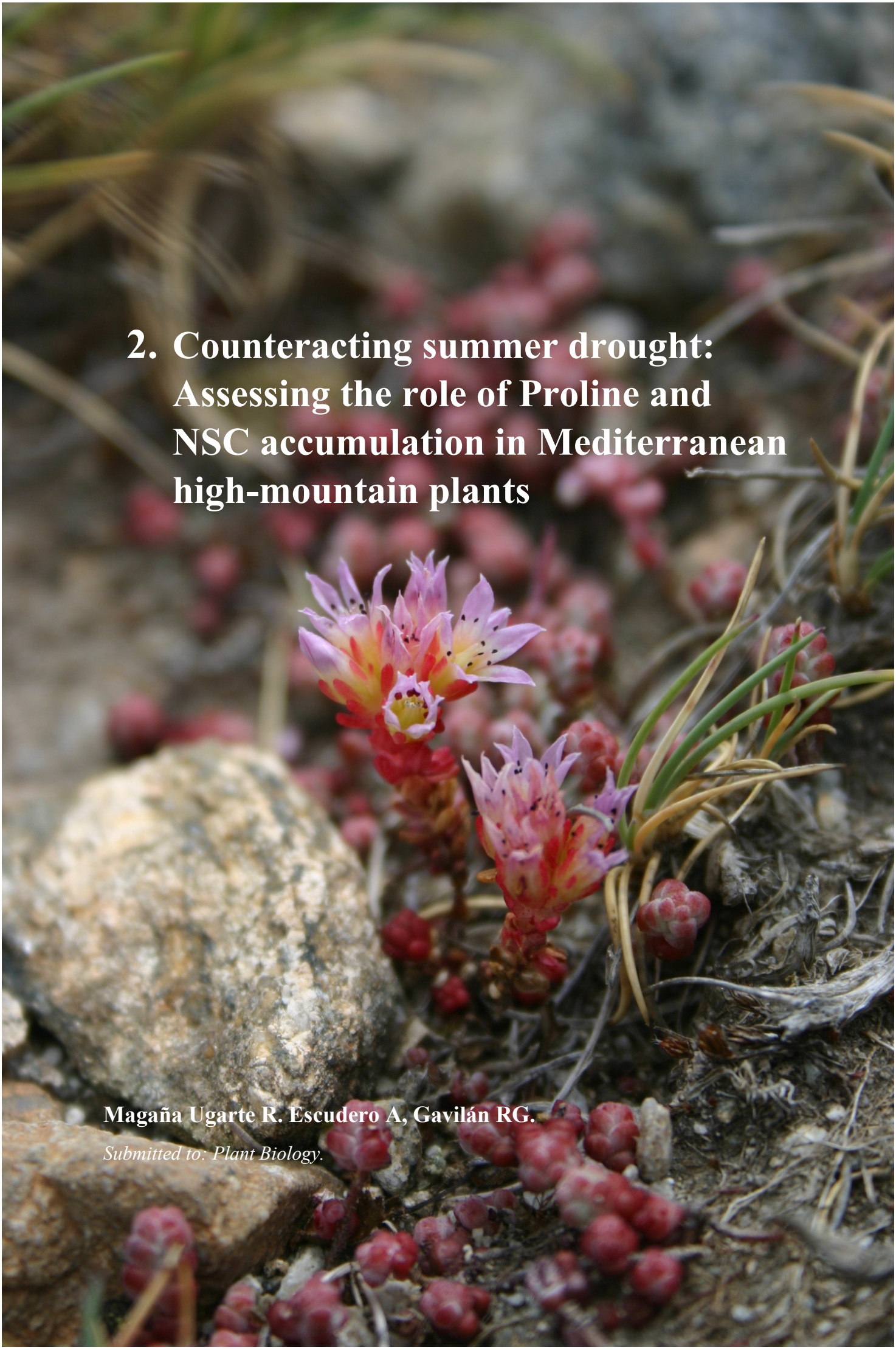


2. Counteracting summer drought: Assessing the role of Proline and NSC accumulation in Mediterranean high-mountain plants

Magaña Ugarte R. Escudero A, Gavilán RG.

Submitted to: Plant Biology.

ABSTRACT

- In Mediterranean high-mountains, the constraining weight of summer drought over plant life feasibly derived in a series of adaptations to ameliorate its impact on plant performance, for instance osmolyte accumulation. However, such specific mechanisms remain unknown despite the challenge that summer drought poses over these specialists, most of them being gravely imperiled by the ongoing climate change.

- Consequently, we aimed to uncover the role of well-known osmoregulators proline and non-structural carbohydrates (NSC), as pointers of the drought-stress response in seven high-mountain specialists, representative of the high-mountain flora in Central Spain, throughout an altitudinal gradient.

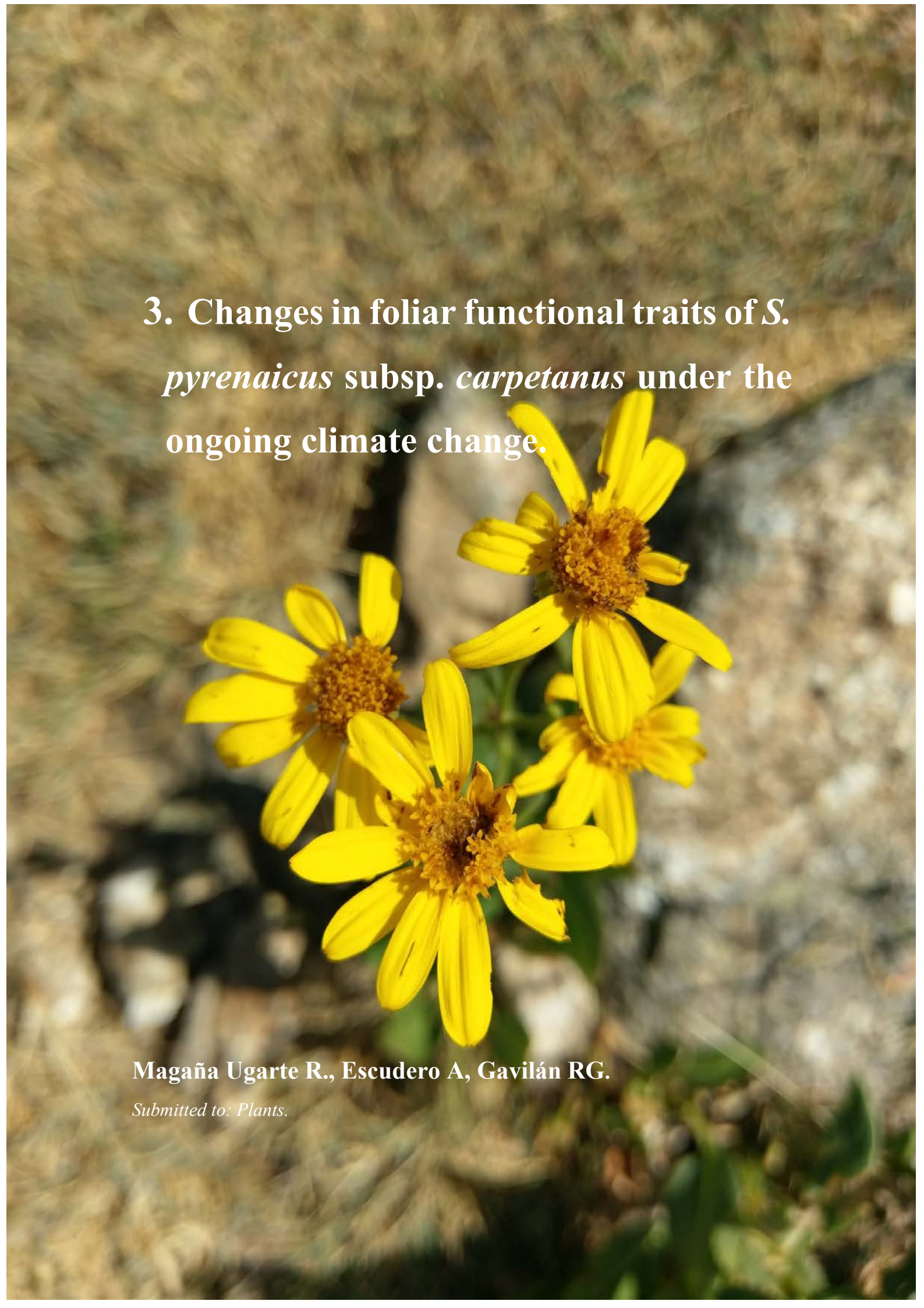
- Results revealed the key role played by osmolyte accumulation countering summer drought, with proline as the main osmolyte involved in the drought-stress response in the studied specialists and NSC buildup contributing in the summer drought response in a coarser growing season. Hence, the dual action of NSC and proline to provide optimal drought endurance in these species is inferred.

- Additionally, despite the high resilience of these species to summer drought underlined in our findings, their significant responsiveness to extreme climate episodes revealed the potential liability of Mediterranean high-mountain communities to the impending coarsening of summer stress, as consequence of climate change.

Keywords: Drought, Mediterranean high-mountain, proline, non-structural carbohydrates, resilience.

Nomenclature follows the guidelines in Flora Iberica (Castroviejo et al. 1986) and Flora Europaea (Tutin, 1976).

3. Changes in foliar functional traits of *S. pyrenaicus* subsp. *carpetanus* under the ongoing climate change.



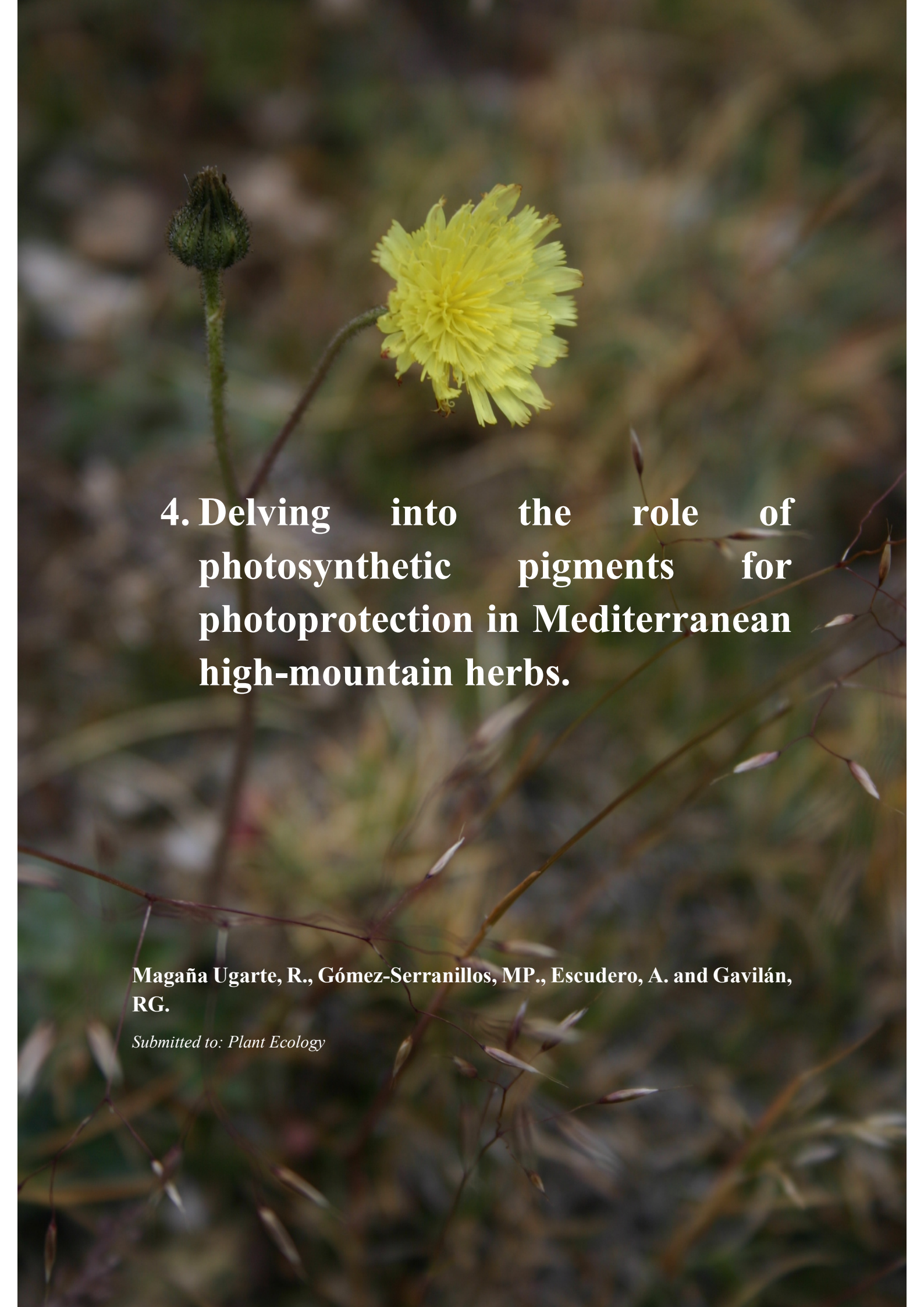
Magaña Ugarte R., Escudero A, Gavilán RG.

Submitted to: Plants.

ABSTRACT

Stomatal behavior and patterning (i.e. distribution, density and size) respond to a series of environmental and plant stimuli, resulting crucial for defining the physiological performance of leaves. Thus, assessing phenotypic modifications in stomatal traits in herbarium individuals arises as an eco-physiological approach to predict how the rising trend of warmer, drier summers could affect plant fitness; particularly in mountain areas already experiencing the effects of climate aggravation, such as Mediterranean high-mountains. We analyzed the variations in foliar macro and micro-morphological traits in *Senecio pyrenaicus* subsp. *carpetanus*, i.e. leaf area and width, stomatal density (SD) and size (SS), from Sierra de Guadarrama in Central Spain over the past 71 years using herbarium specimens. Our findings revealed decreasing trends in leaf width and SS, coupled to an increasing tendency in SD, all correlated with the recent 30-year climate exacerbation in these summits. This evidenced a positive selection favoring traits that allow safeguarding plant performance under drier, hotter weather. Moreover, the opposite responses in SD to climatically contrasting growing seasons (temperate vs. drier climate) suggests the proposed morphology-fitness relationships are mediated by the environmental conditions specific to each growing season. The strong correlation between stomatal traits and climatic variables upholds the role of stomatal patterning in sensing environmental cues in this species, feasibly optimizing the physiological responses involved in the growth-water loss trade-off. The transition to smaller, densely packed stomata observed in recent decades could indicate local-adaptive plasticity in this species to enhance stomatal response, coupled to a potential improvement of WUE, as coarser environmental conditions take place in Sierra de Guadarrama.

Keywords: *stomatal density; leaf traits; high-mountains; adaptive capacity; climate change.*



4. Delving into the role of photosynthetic pigments for photoprotection in Mediterranean high-mountain herbs.

Magaña Ugarte, R., Gómez-Serranillos, MP., Escudero, A. and Gavilán, RG.

Submitted to: Plant Ecology

ABSTRACT

Photosynthetic pigments have been widely studied in lowland species exposed to diverse sets of conditions. However, little is known about their function and bioactivity in plants from high-mountains, particularly in those with Mediterranean-type bioclimate growing under remarkably high UV-B loads, high temperatures and drought stress. Herbaceous Mediterranean high-mountain specialists among the dominant components of grasslands, were collected across an altitudinal gradient to analyze the diurnal and seasonal variation of photosynthetic and photoprotective pigments, i.e. chlorophyll, carotenoids and xanthophyll, in consecutive and climatically contrasting years.

Significant differences in the functioning of the xanthophyll cycle were found among species, either acting as a sustained photoprotective strategy or triggered only under coarser summer stress. Furthermore, we detected increases with altitude in the xanthophyll cycle pool, enhancing in hot growing seasons with low soil water availability, whereas maintaining high values in low-elevation samples irrespective of seasonal climate. The high lutein levels found in all species at contrasting elevations, suggested the involvement of the lutein cycle providing "sustained" photoprotection in these specialists. The aforementioned, coupled to the minor variances in chlorophyll *a/b* ratio with altitude and seasonal aggravation of summer stress, indicate xanthophyll cycle pigments are essential for photoprotection in high-mountain plants, allowing them to fulfill their growing cycles and heightening their function under more adverse conditions befalling their growing season.

Keywords: Mediterranean high-mountain plants, chlorophyll, xanthophyll cycle, photoprotection, summer stress.

GENERAL DISCUSSION

GENERAL DISCUSSION

The combination of increasing climatic stresses that dictate plant life in high-mountain environments provides a diversity of microclimates within small scales through altitudinal gradients, simulating macroclimatic interactions. Similar to other mountainous regions (e.g. alpine), Mediterranean high-mountains are exposed to halting events and low temperatures throughout most of the year. However, they differentiate due to the drought period befalling in summertime, which coincides with the brief active growing season of vegetation in these habitats (Cavieres et al., 2000; Giménez-Benavidez et al., 2007; Gutiérrez-Girón and Gavilán, 2013). This denotes that the establishment and performance of Mediterranean high-mountain vegetation is influenced by two severity gradients acting in opposite directions: the low temperature stress increasing with altitude, and drought stress intensifying as altitude decreases (Pescador et al., 2015).

The predicted changes in temperature and rainfall regimes in Mediterranean high-mountain areas, coupled to the upward shift of the tree line all as outcomes of climate change, underline the potential liability of these species to the impending aggravation of climate (Giorgi and Lionello, 2008; Gutiérrez-Girón and Gavilán, 2013). Hence, studying the variations in morphological and physiological traits in the individual species conforming these plant communities will enable the identification of attributes vital for conferring resilience to the consequences of climate change. Or, in consequence, help identify those more vulnerable to climate worsening and foster the adoption of measures to promote their conservation.

The direct relationship between leaf traits with carbon gain, nutrient loss and water usage in plants, connotes their study could allow identifying and estimating plant performance in high-mountain habitats under coarser climate (Yang et al., 2011; Gornish and Prather, 2014; Gutiérrez-Girón et al., 2015). In this manner, in the present thesis a series of stress indicators were evaluated on Mediterranean high-mountain specialists, based on their establishment as monitors and markers of stress responses among lowland and woody species under concurrent abiotic pressures. Moreover, the specific responses to the main driver of plant life in Mediterranean high-mountains disclosed in this thesis, i.e. summer stress, adds to the refinement of conservation strategies of these plant communities, contributing to the offsetting of the ongoing biodiversity and climate crises and ultimately closing the current great knowledge-gap between alpine and Mediterranean high-mountain vegetation. The following response mechanisms and well-known stress indicators were studied, aiming to refine the stress-specific responses elicited in Mediterranean high-mountain specialists to summer stress: we assessed osmotic adjustment via

the quantification of osmolytes Pro and NSC buildup, assuming it as a key mechanism in Mediterranean high-mountain orophytes to counteract the effects of dehydration stress (imposed by both summer drought and the occasional halting events befalling the growing season); and further characterize the degree of liability of the studied orophytes to the impending reduction in rainfall patterns. We also studied the seasonal variation in chlorophyll content and quantified the diurnal variation of the xanthophyll cycles, associated with drought stress incidence and the enhancement of the photosystem under rapidly wavering diurnal climate, respectively. In addition, an assessment of the changes in morphological and micro-morphological leaf parameters was performed, in order to pinpoint the adaptive capacity of pioneer species *S. carpetanus* to the recent climate aggravation registered in Sierra de Guadarrama, and associate these with the ongoing trend of increasing temperatures and scattered rainfall regimes.

The findings of this PhD thesis provide evidence that Mediterranean high-mountain plants have evolved and optimized their life strategies to survive the prevailing severe conditions in these habitats, eliciting different enduring mechanisms when the extreme limit, i.e. threshold, for an environmental variable is exceeded. The different responses observed between climatically contrasting years in each species reinforced the idea that the accumulation of Pro and NSC in Mediterranean high-mountain plants is strongly associated with the length and severity of drought events. However, contrary to the initial hypothesis, we detected a lack of local adaptation to summer drought in low-altitude populations of herbaceous species, given the absence of significant differences in osmolyte buildup between populations from contrasting altitudes.

Nevertheless, significant differences in the degree of sensitivity to seasonal reduction of SWC were found among the studied species, allowing their arrangement into two groups: 1) Resilient species, denoting a higher degree of responsiveness to small variations in SWC and the ability to pull through severe stress via a significant buildup of Pro or NSC, i.e. *E. penyalarense, H. vahlii* and *J. humilis*; or 2) Robust species, triggering a substantial response only under severe climatic events or stark soil water depletion, i.e. *F. curvifolia, J. alpina, S. carpetanus*. The hardiness to climate extremes detected in the chamaephytes is conveyed by its atmosphere-decoupling capacity allowing the maintenance of more steady temperatures and humidity within their canopy, compared to their surroundings (Körner, 2003). In regards to *S. carpetanus*, the denoted drought-hardiness may be linked to long-term feedbacks driven by longstanding environmental coarsening in Sierra de Guadarrama, which has allowed the selection of functional traits granting greater drought endurance in this species (Copeland et al., 2016; Magaña Ugarte et al., in press); while in *F. curvifolia* it is attributed to its dominance and strong competitiveness in these xerophytic pastures (Pescador et al., 2014).

In addition, our findings revealed how drought acts by imposing certain limits in these orophytes, which elicited a response when subject to a particular level of dryness. For instance, Pro accumulation differing in *J. humilis* between climatically contrasting years, being significantly higher when experiencing more severe conditions as in 2017 allowing the extension of its growing season, compared to its diminished accumulation in a milder growing season (i.e. 2018).

As mentioned before, in Mediterranean high-mountains the sporadic freezing events during the active growing season gives rise to the concomitant effect of drought and freezing stress in the studied specialists (Pescador et al., 2016; Valladares et al., 2004). Our results evidence the convergence between drought and low temperature stress responses, revealed in the enhanced inulin and Pro accumulated in *E. penyalarense* and *F. curvifolia* in response to summer drought. This substantial osmolyte buildup further justify their previously reported enhanced freezing resistance to halting events befalling the growing season (Pescador et al., 2016) (Chapter 2). Furthermore, and albeit in the absence of clear accumulation patterns for most NSC quantified, the seasonal maintenance of high inulin levels in herbs from contrasting elevations in the studied altitudinal gradient, and its heightening under severe summer stress (i.e. 2017), advocate for its role in enhancing drought in the studied herbaceous species. The previously mentioned, in addition with the seasonal raffinose buildup in *E. penyalarense* and *S. carpetanus* during the atypically dry growing season of 2017, allows inferring NSC and Pro are not mutually-excluding mechanisms that, in the event of severe summer stress, act simultaneously to optimally counteract summer stress in these high-mountain species.

Overall, these findings indicate Pro as the primary osmoprotectant involved in the drought-freezing stress response in the studied Mediterranean high-mountain specialists, whose accumulation is driven by the intensity and frequency of these pressures during the growing season. The observed proclivity for Pro accumulation in these orophytes over NSC buildup could be related to an adaptation strategy associated with the briefness of the growing season in these habitats; aiming to focus photosynthate investment into primary functions such as tissue growth and reproduction rather than into osmoregulation.

In herbs, species-specific habitat adaptations are generally associated with their annual growth schemes, especially in those with shorter life spans (Liu et al., 2019). In Mediterranean high-mountain areas, the appearance of unsuitable habitats altering species occurrence patterns and performance, outcome of seasonal SWC reduction, feasibly explains the observed withdrawal from the area of study of *E. penyalarense* following recurrent heatwave episodes (Chapter 2, Fig. S1). The higher osmolyte accumulation elicited under scarce SWC, significant enhancement in the VAZ pool, low Chl_{TOTAL} and scarce individuals appearing in a dry, hotter growing season (i.e. 2017, 2019); contrast the smaller VAZ pool, lower osmolyte buildup, higher Chl_{TOTAL} and species

abundance observed in a milder season (i.e. 2018), denoting the constraint that graver summer stress poses over this species. The drought-mediated occurrence of the endemic species *E. penyalarense* described in Chapters 2 and 4, emphasizes the susceptibility of this narrow-endemic to severe summer stress; and can be extrapolated to predict its vulnerability to the impending lengthening and exacerbation of summer stress in these mountains (Nogués-Bravo et al., 2008). Similarly, *S. carpetanus* denoted a withdrawal from its growing sites following heatwaves and when subject to scant SWC. However, contrary to the observed in *E. penyalarense,* followed by its sporadic re-sprouting once the environmental conditions became more suitable (Chapter 2, Fig. S1). The observed behavior in *S. carpetanus* could be related to its life history, regarded as a vital factor shaping trait differentiation and functional ecology of annual species; specifically, in those described as "subordinate". This "subordinate" nature enables *S. carpetanus* to thrive in similar niches throughout a variety of habitats and associate with a diversity of dominants due to a high degree of homeostasis under unstable or limiting environments (Grime et al., 2007; Liu et al., 2019). Thus, the ability for re-sprouting after being subject to adverse conditions, reinforces the key role of detected local adaptations in *S. carpetanus* to climate aggravation in these mountains via the optimization of the physiological responses involved in the growth-water loss trade-off, such as the adjustments in leaf morphology (i.e. stomatal patterning and leaf size) described in Chapter 3 and in the following paragraph.

The confirmed trend of rising temperatures during the growing season in Sierra de Guadarrama, coupled to the wavering and reduction of rainfall patterns, compels plants to adjust their physiology to tackle both water loss and avoid leaf overheating to avoid damage to their structures and potential photoinhibition. Adjustments in SD and SS can be the result of the effect of genetic and environmental factors to which the plant is subject during its growing season (Bertolino, et al. 2019). The transition to smaller, densely packed stomata registered in *S. carpetanus* in recent decades could indicate a local-adaptive strategy in this species to optimize stomatal response and enhance water-use-efficiency to counterbalance more adverse environmental conditions. Assuming ecological trends as the greater driver of adaptive behavior of stomata (Deans et al., 2019; Körner et al., 1989), the observed adjustments in SD and SS in *S. carpetanus* can be interpreted as the outcome of a long-term exposure to adverse conditions. Furthermore, the observed substantial decrease in LA and SS and higher SD in response to the escalation of summer stress in these mountains over the last 30 years, indicates a positive selection of traits enhancing plant performance under drier, warmer weather (Carlson et al., 2016). This emphasizes the higher adaptation capacity of this species to the impending climate exacerbation in Sierra de Guadarrama. However, further studies encompassing gas-exchange parameters and nutrient status are encouraged to determine whether these adjustments are sufficient to sustain efficient photosynthetic activity and estimate their maximum thresholds to drought and heat stress.

Incorporation of these analyses will provide a holistic understanding of the ecophysiology in *S. carpetanus*, coupled to accurately assessing the vulnerability of the structure and dynamics of these high-mountain communities, in the context of climate change.

Along with evaluating the morphological adaptations and their association with optimal resource uptake and usage under challenging conditions in these specialists, one should consider investigating the variations in the phytochemicals allowing them to perform and fulfill these primary strategies. This given the thermolabile nature of photosystems and the susceptibility to enhanced photosynthetic ROS generation and buildup under abiotic stresses; which could result in decreased photosynthesis and, thus, carbon fixation (Streb et al., 2003; Peñuelas and Munné-Bosch, 2005). Thus, assessment of photosynthetic pigment composition can be employed as an indicator of plant physiological status providing insight on the nutrient status and general stress, coupled to denoting the photosynthetic efficiency and dissipation of excess energy captured (Spitaler et al., 2008; Zidorn 2010).

Increased de-epoxidation of the VAZ xanthophyll cycle heightened the stability of the photosynthetic apparatus in alpine plants subject to simultaneous drought and heat stress, through thermal dissipation of excess energy and highly efficient ROS scavenging capacity (Abeli et al., 2015; Buchner et al., 2017; Buchner et al., 2015). Accordingly, we observed the fundamental involvement of the VAZ cycle and lutein in the photoprotection of the studied high-mountain herbs in response to summer stress; with significant differences in their contribution among species (Chapter 4). In species inhabiting above 2,100 m such as *H. vahlii* and *J. humilis*, we noticed a continued photoprotection ascribed to a significant diurnal increase in the de-epoxidation of the VAZ cycle, enhancing the stability of photosynthesis via NPQ_f and the stabilization of PSII reaction centers to recurring abiotic pressures (Buchner et al., 2015). Conversely, the significant differences in the activation patterns of the VAZ xanthophyll cycle in the remaining studied herbs suggested its activation in these species is only triggered under severely challenging summer stress. Nevertheless, both strategies seemingly confer a good extent of photoprotection under coarser summer stress befalling the growing season; based on the increased rate in de-epoxidated compounds Z and A in all species from high and low elevations in the drier growing season of 2019 (43-60% and 50%, respectively). These findings also denote the absence of local adaptations in this mechanism, indicating NPQ_f strategies in these species are mediated by the severity of the conditions befalling the growing season and not local microclimate (Bascuñán-Godoy et al., 2010).

Additionally, this study revealed the potential involvement of an additional photoprotective strategy in these specialists: the less-studied Lx cycle; given the constantly high Lut levels found in all species from contrasting altitudes. In turn, we propose the Lx cycle as an additional,

sustained energy-dissipation mechanism, functioning regardless of the severity of the climatic conditions incident in the growing season (García-Plazaola et al., 2003; Matsubara, et al. 2001). Moreover, the overall lack of seasonal changes in the Chl *a/b* ratio as summer drought increased, advocates for the greater endurance of the photosynthetic apparatus in these specialists to counteract summer stress, conveyed by the significantly high DEPS. This denotes the activation of photoprotective mechanisms, i.e. xanthophyll cycles both VAZ and lutein, as crucial to guarantee the maintenance of an optimal functioning of the photosystem in these orophytes subject to challenging growing conditions. Nonetheless, given the foreseen coarsening of summer stress in the Mediterranean high-mountains (Nogués-Bravo et al., 2008), further studies are needed to determine the fate and functioning of the photosynthetic system in these orophytes throughout their brief growing season. For instance, incorporating field measurements of the F_v/F_m ratio can be a reliable and rapid indication of changes in the maximum photochemical efficiency of PSII in response to temperature or drought stress, giving an accurate and instant measurement of the inner-workings of the photosystem under specific conditions, time and place (Ritchie 2006).

In view of the ongoing biodiversity and climate crises, the specific responses to the environmental pressures dictating plant life in Mediterranean high-mountains disclosed in this PhD thesis contribute to refining conservation strategies of these plant communities. These findings point out the higher vulnerability of Mediterranean high-mountain grasslands compared to that of shrubby communities; particularly threatening endemic, narrowly-distributed species such as *E. penyalarense* or *J. humilis*. Moreover, the greater constraints for grassland communities to the future temperature increases in these summits is illustrated in the responses of dominant species *F. curvifolia.* The significant reduction in Chl_{TOTAL}, added to the exerted significant buildup of Pro in response to the recurrent heatwaves and drier conditions present in 2017, compared to the milder climate occurring in 2018 (Chapters 2 and 4), points out the plausible vulnerability of these habitats to higher temperatures and, by extension, the aggravation of summer stress.

Overall, despite the assortment of protective mechanisms displayed by these herbaceous specialists (i.e. high accumulation of osmolytes and photoprotective pigments), their sensibility to more adverse summer stress indicates a latent threshold for these counteracting responses (Chapter 2, 4), impending to be surpassed in the event of more recurrent heatwaves and/or the lengthening of the summer drought period (Nogués-Bravo et al., 2008). In addition, the earlier snow melting reported in recent years in these mountains, suggests the aggravation of a major environmental pressure on the growing season of these specialists: water availability. The combination of earlier thawing with reduced and more scattered rainfall patterns in the period for plant development, together with the occurrence of freezing events in these mountains, could foster an enhanced dehydration stress in high-mountain specialists; particularly during their brief

growing season. These potentially translating into adjustments or modifications of species patterns, community dynamics and, to a greater extent, the structure at both local and broader scales.

CONCLUSIONS

- Appraisal of plant responses and potential adaptations to concomitant abiotic stressors via functional traits, established as indicators of phenology, nutrient status and general plant stress in alpine flora, could foster closing the profound knowledge gap between alpine and Mediterranean high-mountain vegetation.

- Implementing these well-known stress indicators will enable the comparison between plant responses among mountainous regions, along with more accurate estimations of plant fitness in high-mountain habitats in view of the ongoing climate exacerbation.

- Osmolytes Proline (Pro) and non-structural carbohydrates (NSC) occurred as complementing strategies playing a key role in the response of Mediterranean high-mountain orophytes to more severe summer drought; with Pro emerging as the primary osmoprotectant entailed in the drought-freezing stress response of these orophytes, particularly in those denoting greater sensitivity to minor changes in SWC, e.g. *E. penyalarense*. The observed proclivity for Pro accumulation over NSC buildup could be attributed to an adaptation strategy linked to the briefness of the growing season in these habitats, optimizing photosynthate investment into primary functions rather than into osmoregulation.

- Summer drought can enforce and push the response-limits in high-mountain specialists, displayed when species experience a certain level of dryness. For instance, the significantly high Pro accumulation elicited in *J. humilis* to subsist an atypically dry and warm growing season; associated to the extension of the growing season in this species to overcome a feasible drought-induced nutrient deficiency.

- The enhanced inulin and Pro accumulated in *E. penyalarense* and *F. curvifolia* in response to summer drought evidences the clear convergence between drought and low temperature stress responses. This substantial osmolyte buildup further justifies their documented enhanced freezing resistance to halting events befalling the growing season.

- The observed adjustments in leaf and stomatal morphology in *S. carpetanus* denote a longstanding, positive selection of traits enhancing plant performance in response to the shift towards drier and coarser conditions during the growing season in Sierra de Guadarrama; underlining its higher adaptation capacity to the impending climate exacerbation in these mountains.

- Species occurrence patterns detected in *S. carpetanus* subject to highly constraining environmental conditions (i.e. local withdrawal under extreme climatic events or scarce SWC with its subsequent re-appearance) could be related to its life history as an annual "subordinate" species of these high-mountain communities. The ability for re-sprouting after being subject to adverse conditions reinforces the observed strategic local adaptations in herbaria specimens: decreasing tendencies of LA and SS and a higher SD in response to the escalation of coarser growing seasons in these mountains over the last 30 years.

- Contrasting the low de-epoxidation of the xanthophyll cycle under milder conditions, the greater DEPS observed in *E. penyalarense, F. curvifolia, H. vahlii* and *J. humilis* from contrasting elevations when subject to coarser summer stress (i.e. 2019) suggests a potential adaptive strategy to avoid photoinhibition via higher thermal dissipation; shaped by the severity of the conditions befalling each growing season and not local microclimate.

- In view of the consistently high lutein levels found in all herbaceous species from high and low elevations, these findings disclose the potential involvement of the less-studied Lx cycle arising as an additional, sustained energy-dissipation mechanism, activated irrespective of the environmental conditions befalling the growing season.

- The overall absence of seasonal changes in the Chl *a/b* ratio in response to summer drought aggravation indicates the greater endurance of the photosynthetic apparatus in these high-mountain specialists to counteract summer stress conveyed by the activation of photoprotective mechanisms, i.e. xanthophyll cycles both VAZ and lutein, safeguarding the optimal functioning of the photosystem in these species growing under challenging environments.

- The drought-mediated species occurrence of the narrow-endemic *E. penyalarense,* coupled to the significant enhancement of osmolyte accumulation and the VAZ pool under drier, warmer growing seasons, underlines its greater vulnerability to the foreseen increase in seasonality and incidence of extreme climatic events during these periods. In addition, it avows the existence of a "ceiling response" to summer drought, plausibly inherent in other high-mountain specialists; envisaging alterations in species patterns, community dynamics and structure of Mediterranean high-mountain communities' outcome of climate exacerbation.

APPENDIX

APPENDIX

LIST OF ABBREVIATIONS, IN ALPHABETICAL ORDER

- δ^{13}**C**, Carbon-13; a natural, stable isotope of carbon with a nucleus containing six protons and seven neutrons.
- **A**, antheraxanthin
- ***Cab/xc* ratio**, total chlorophyll (Chl_a + Chl_b) vs. total carotenoids ratio
- **Chl**, chlorophyll
- **Chl** $_a$, chlorophyll *a*
- **Chl** $_b$, chlorophyll *b*
- **Chl *a/b*,** chlorophyll a/chlorophyll b ratio
- **Chl**$_{TOTAL}$, total chlorophyll (Chl_a + Chl_b)
- **DEPS**, de-epoxidation state of the VAZ xanthophyll cycle (%)
- **DW**, dry weight of plant material (g or mg)
- F_v/F_m, maximal photochemical efficiency of photosystem II
- **FW**, fresh weight of plant material (g or mg)
- **GLMs**, generalized mixed models
- g_s, stomatal conductance. Rate at which a gas diffuses through the stomatal aperture.
- **LA**, leaf area (cm^2)
- **LMA**, leaf mass area (g or mg)
- **LNC**, leaf nitrogen content
- **Lut**, lutein
- **Lx**, Lutein epoxide cycle
- **N,** nitrogen
- **NPQ**, non-photochemical quenching
- **NPQ**$_f$, fast relaxing component of NPQ (i.e. xanthophyll cycles)
- **NPQ**$_s$, slow relaxing component of NPQ
- **NSC**, non-structural carbohydrates
- **PFTs**, plant functional traits
- **Pro**, Proline
- **PSII**, photosystem II.
- **R**$_0$**,** number of days in a month without registered rainfall
- **Rainfall** $_{TOTAL}$, mean monthly rainfall (mm/month)
- **ROS**, reactive oxygen species
- **SD**, stomatal density (number of stomata per mm^2)
- **SLA**, specific leaf area (leaf area/leaf $_{DW}$; cm^2/g)

- **$Snow_{DAYS}$**, average of days that registered snow within a month
- **SS**, stomatal size (μm)
- **Sun_{MEAN}**, mean daily insolation per month (hr/day)
- **SWC**, top-soil water content (%, by vol.)
- **T_{MAX}**, maximum mean air temperature (ºC)
- **T_{MED}**, mean air temperature (ºC)
- **T_{MIN}**, minimum mean air temperature (ºC)
- **V**, violaxanthin
- **VAZ cycle**, violaxanthin-antheraxanthin-zeaxanthin cycle = VAZ cycle = VAZ xanthophyll cycle
- **VAZ pool**, total compilation (pool) of antheraxanthin, violaxanthin and zeaxanthin.
- **WUE**, water-use efficiency. Photosynthetic water-use efficiency refers to the ratio of carbon assimilation vs. transpiration; whilst water-use efficiency of productivity, is defined as the ratio of biomass produced to the rate of transpiration.
- **Z**, zeaxanthin